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THE UNIVERSITY OF ALBERTA

STUDIES ON THE PROBLEMS ASSOCIATED WITH THE UTILIZATION OF RAPESEED MEAL BY POULTRY

by

© YEOW KWANG GOH

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
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ABSTRACT

A series of experiments (Part I) were undertaken to study the use of dye-binding for estimating the quantity and quality of protein in rapeseed meal (RSM) as well as the degree of protein denaturation caused by heat treatment. Results obtained from Experiment 1 in which the dye-binding capacities (DBCs) with Acid Orange 12 and the crude protein contents (N x 6.25) of 15 samples of RSM were investigated showed that the two parameters were related by the linear equation, $Y(N \times 6.25) = 2.2 + 0.27X$ (DBC) with a correlation coefficient of 0.98. The equation was tested for its applicability to 126 RSMs in Experiment 2 and it was found that it under-estimated or over-estimated the crude protein of about 20% of the samples by 1% or more. The deviation was caused mainly by atypical content of basic amino acids, particularly of lysine, in these meals. On the other hand, the dye-binding capacities of the protein (DBCPs) of 21 samples of RSM were found to correlate highly to the lysine and available lysine contents of the meals ($r = 0.84$ and 0.79) indicating the potential of the use of DBCP as a protein quality index for RSM.

In Experiment 3, moist heat treatment was found to decrease the DBCP of RSM as a function of autoclaving duration at 121°C. Protein quality, estimated by available lysine determination, showed a parallel but accelerated decline compared to the decrease in DBCP noted.

ABSTRACT

A series of experiments (Part I) were undertaken to study the use of dye-binding for estimating the quantity and quality of protein in rapeseed meal (RSM) as well as the degree of protein denaturation caused by heat treatment. Results obtained from Experiment 1 in which the dye-binding capacities (DBC) with acid orange 12 and the crude protein contents (N x 6.25) of 15 samples of RSM were investigated showed that the two parameters were related by the linear equation, %CP (N x 6.25) = 2.2 + 0.407 (DBC) with a correlation coefficient of 0.98. The equation was tested for its applicability to 125 RSMs in Experiment 2 and it was found that it under-estimated or over-estimated the crude protein of about 20% of the samples by 1% or more. The deviation was caused mainly by atypical contents of basic amino acids, particularly of lysine, in these meals. On the other hand, the dye-binding capacities of the protein (DBC/g) of 21 samples of RSM were found to correlate highly to the total and available lysine contents of the meals (r = 0.94 and 0.79) indicating the potential of the use of DBC/g as a protein quality index for RSM.

In Experiment 3, moist heat treatment was found to decrease the DBC/g of RSM as a function of autoclaving duration at 121°C. Protein quality, estimated by available lysine determination, showed a parallel but accelerated decline compared to the decrease in DBC/g values.

Results obtained from two chick bioassays (Experiment 4) indicated that commercial RSMs with DBCP values ranging from 335 to 360 mg Acid Orange 12/g protein were comparable from the point of view of growth promotion of male broiler chicks. However, laboratory heat-damaged RSMs which had DBCP values equal to or lower than 327 mg/g protein were found to have inferior growth promoting values as measured by total protein efficiency in bioassays involving male broiler chicks. The degrees of protein denaturation in autoclave and oven-heated RSMs, estimated by the dye-binding method, showed that the meals were more severely affected by heating in an autoclave at 121°C than by heating in an oven at the same temperature for similar periods of time (Experiment 5).

In Part II of the studies, the effect of feeding high and low glucosinolate RSMs (HG-RSM and LG-RSM) at 5 and 10% of the ration to laying hens on the transfer of dietary iodine to eggs was examined. Percent ^{125}I incorporated into eggs during the steady state showed that the amount of ^{125}I transferred to egg yolk was significantly reduced by the inclusion of HG-RSM but not by the LG-RSM ($P<0.05$).

In Part III of the studies, the effects of glucosinolate level in RSM and supplementary choline on the production of fishy eggs by fishy egg layers were investigated. Rapeseed meals with high, medium and low glucosinolate levels (HG-RSM, MG-RSM and LG-RSM) were fed at 10% of the laying ration.

The mean trimethylamine (TMA) level and mean fishy odor score were numerically highest in the eggs produced by the birds fed the ration containing 10% HG-RSM but not significantly different from those resulting from feeding birds the ration containing 10% LG-RSM. Supplementing a laying ration devoid of RSM with choline at 250 and 500 mg/kg ration did not cause the birds to lay eggs with measurable TMA content or detectable fishy odor.

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INTRODUCTION

Rapeseed, first grown in Western Canada for commercial use in 1943, has now become the most important oilseed crop grown in Canada. During recent years its annual production has fluctuated around 1.2 million metric tons (Runciman and Olson, 1975). The seed, containing 40% or more of oil by weight, is grown mainly for its oil. The oil, obtained by extracting the crushed seeds with hexane and further refining, is used as an edible oil. The residue, left after oil removal, is rich in protein and is used as a source of protein for livestock and poultry.

Early attempts to use rapeseed meal (RSM) in rations for livestock and poultry were not very successful owing to the presence of goitrogenic factors in RSM and to the relatively low metabolizable energy value of RSM. As a result, only limited levels of RSM were recommended for use in feeds for poultry and swine (Clandinin _et al._, 1972; Bowland and Bell, 1972).

In recent years, the prospects for increased utilization of RSM by feed manufacturers and for wider acceptance of RSM by livestock producers have greatly improved as a result of developments on two fronts; the production of new varieties of rapeseed of improved quality and an increased world-wide demand for protein for human consumption. With regard to the former, plant breeders have developed varieties of rapeseed which are low in glucosinolate content and are in the process of developing varieties which are low in

fibre content (Downey and Klassen, 1977). In addition, rapeseed processing technology has improved to the point where the quality of the protein in RSM compares favourably with other protein-rich feedstuffs. These advancements have made it possible to double the recommended levels of usage for low glucosinolate RSM by monogastric animals and poultry as compared to the usage levels recommended for high glucosinolate type RSM. On the other front, the world-wide shortage of protein for human consumption has greatly increased the demand for soybean meal which, in the past, has been the major source of protein for livestock and poultry feeds. The scarcity and high price of soybean meal have compelled animal nutritionists to seek alternate sources of protein. As a consequence of the above, it is logical to anticipate improved potential for increased use of RSM by feed manufacturers throughout the world.

Rapeseed meal is, at present, widely used in rations for poultry. However, in spite of its much improved nutritional quality, there are still unsolved problems associated with the use of RSM in rations for poultry (Clandinin _et_ _al_., 1977) which require solution. This thesis is concerned with studies on some of these unsolved problems.

OBJECTIVES OF THE STUDIES

The objectives of the first series of experiments (Part I) were to investigate the feasibility of using the dye-binding method as a rapid and inexpensive procedure for determining the crude protein content of RSM and for eval-

uating the quality of the protein of RSM.

The objective of the second study (Part II) was to determine the effect that feeding low and high glucosinolate type RSMs to laying chickens had on the iodine content of eggs.

The final study (Part III) was designed to determine the factors in RSM which cause layers of brown-shelled eggs to produce eggs that have a "fishy" or "crabby" odor.

PART I

STUDIES ON THE USE OF DYE-BINDING FOR ESTIMATING THE QUANTITY AND QUALITY OF PROTEIN IN RAPESEED MEAL

A Introduction

Numerous so-called rapid methods have been developed as alternatives to the conventional macro-Kjeldahl procedure for measuring the protein content of cereal grains, oilseed meals and other biological materials. The principles on which these methods were based were completely different from those of the Kjeldahl method but in most cases were calibrated against it. They include; the measure of gaseous nitrogen derived from protein (Dumas' method), reaction of peptide chains of protein with reagents to form color complexes followed by spectrophotometric determination (Biuret method) and infrared absorption by peptide linkages between amino acids of protein molecules (IR method) etc. In spite of the advantages of these analytical procedures over the Kjeldahl method, most of them have only been used in research studies and only a limited number have been adopted officially by the Association of Official Analytical Chemists (A.O.A.C., 1970). One method which shows great potential and which meets the criteria of being rapid and inexpensive and which seems to be gaining in popularity is the dye-binding method. It was thought that by careful standardization and proper refinement of the analytical procedure, that the method might prove useful for daily quality control work in rapeseed processing plants and feed mills.

B Review of Literature

a Historical Development of the Method

It has long been known that acidic and basic dyes were effective protein precipitants. However, it was the persistent error observed in titration studies of protein solutions that caused Chapman et al. (1927) to measure the amount of certain acid dyes which were bound by proteins at various pH values. The proteins used in their studies were mainly isolated proteins such as gelatin, casein and fibrin. The complex nature of the dye-protein binding reaction was later studied by Rawlins and Schmidt (1929, 1930). They concluded that the interaction taking place between the dye ions and the oppositely charged ions of proteins was of chemical nature and reacted in stoichiometric proportions.

The technique of dye-binding, in its earliest stages of application, was used mainly for studying the mode of protein interactions with small molecules in living systems (Klotz, 1953). More recently, the procedure has been used to estimate the quantity and quality of protein.

b Theoretical Basis of Dye-Binding for Estimating Crude Protein Content

Nearly all of the dye-binding procedures described in the literature employed an acid azo dye and originated from the method devised by Frankel-Conrat and Cooper (1944). The principle factor involved in the reaction is the electrovalent attraction between the dye anions and the protein cations. The positively charged groups of the protein arise from the three basic amino acids, namely the epsilon amino

group of lysine, the imidazole group of histidine and the guanidine group of arginine and to a less extent the terminal groups of peptide chains. It would seem reasonable to expect a direct stoichiometric relationship between the amount of dye bound and the number of basic groups in the proteins of the sample. The amount of dye bound by the proteins of the sample should, therefore, serve as a measure of the total basic amino acids in the sample. If the amino acid composition of the protein of a biological material remains constant, the amounts of dye bound by samples of the material should be a function of their total protein contents. It is because of this theoretical potential and because of the practical advantages of the procedure that the method has been extensively studied and tested for its application in the determination of the total protein content of numerous foods and feedstuffs.

c Outline of the Method

Although experimental conditions selected as well as the kind of azo dye and equipment used might vary from one procedure to another, the basic technique is similar and may be summarized as follows:

1. The sample, finely ground if obtained in solid form, is thoroughly mixed with a dye solution of known concentration buffered at approximately pH 2.
2. During an equilibration period which may take a few minutes or several hours, the dye and proteins of the sample react to form insoluble complexes.

3. A clear supernatant is obtained by filtration or centrifugation.

4. The absorbance of the supernatant, which contains the unbound dye, is measured either after dilution or directly in a cuvette with a very short light-path length (0.2 mm).

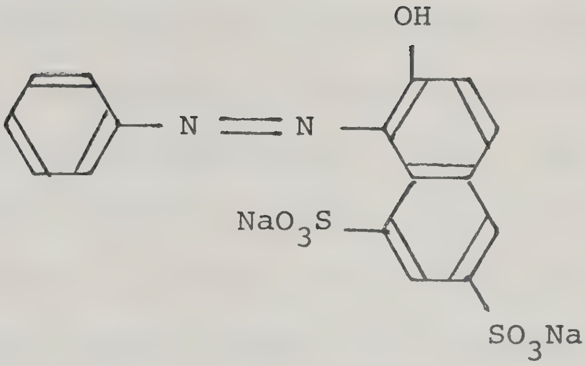
5. The protein content of the sample is then obtained by referring the absorbance or other dye-binding readings to a pre-established calibration curve prepared for that particular protein material.

d Kinds of Dyes Used

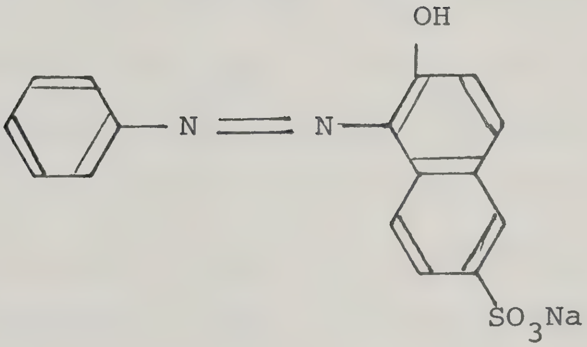
The acid azo dyes commonly used in dye-binding procedure are Orange G (C.I. 16230*), Acid Orange 12 (C.I. 15970) and Amido Black 10B (C.I. 20470). The chemical structures of these dyes are shown in Fig. 1. The difference in dye-binding capacities of these dyes is due to the number of binding sites per molecule. While one molecule of Orange G or Amido Black 10B will bind with two positively charged groups of the protein, one molecule of Acid Orange 12 will bind with only one positively charged group of the protein since there is only one binding site in its molecule. However, it appears that there are no set criteria for the choice of the kind of dye used in the determination. When the procedure was first suggested by Frankel-Conrat and Cooper (1944), Orange G was the only dye investigated for its application in the protein determination of foods and feedstuffs (Udy, 1954, 1956a, 1956b; Moran _et al._, 1963). The azo dye Acid Orange

*Standard color index number.

Orange G (C. I. 16230)


OH
N
N
NaO3S
SO3Na

Acid Orange 12 (C. I. 15970)



Amido Black 10B (C. I. 20470)

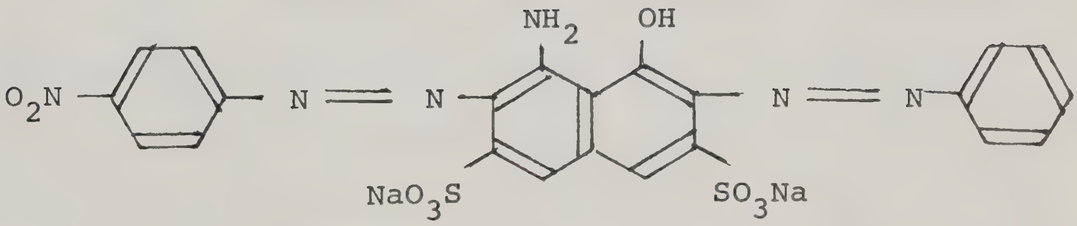

O2N
N
N
NH2
OH
N
N
NaO3S
SO3Na

Fig. 1. Acid azo dyes commonly used in dye-binding studies.

12 was first used by Udy in 1959 (cited by Udy, 1971) and was later found to react well with most commodities investigated. Since then, the use of this azo dye has become preferable over Orange G except in the case of determinations involving herbage (McKenzie, 1976). The use of Amido Black 10B has been limited, on the other hand, to crude protein determinations of dairy products (Dolby, 1961; O'Connell, 1970).

Several non-azo dyes have also been used for binding with proteins to assess protein quantity or quality. They have included phenolphthalein, cresol red, remazol blue and bromophenol blue (Frolich, 1954; Olomucki and Bornstein, 1960; Swaminathan et al., 1973; Hurrell and Carpenter, 1975). The theoretical basis on which these dyes operate in predicting protein quantity and quality is not known.

e Mechanism of Dye-Protein Binding Interaction

The chemical nature of the dye-protein reactions are complex and involve multiple equilibria. Theoretical and mathematical relationships in dye-protein interactions have been reviewed by Rosenberg and Klotz, (1960).

The primary mechanism in the dye-protein interaction is an electrovalent association between the dye anions and cation sites on the proteins. Other than the primary mechanism, several possible secondary binding reactions may also be involved. These secondary bindings may be caused by interactions of the following nature; by hydrogen bonding between dye molecules and proteins, by hydrophobic bonding between dye molecules and proteins and by association between

free dye molecules and other dye molecules already bound to proteins. The binding of dye molecules to protein via the secondary mechanisms is undesirable since the extra dye so bound will over-estimate the crude protein content of the material under assay.

Of the two dyes, Orange G and Acid Orange 12, Orange G has been found to bind with protein primarily via electrovalent force and less via secondary binding than Acid Orange 12 (Lakin, 1973). In fact, the stoichiometric relationship between Orange G bound to the basic groups of several isolated proteins has been demonstrated (Frankel-Conrat and Cooper, 1944) whereas such relationship has, as yet, not been shown for Acid Orange 12. On the other hand, Acid Orange 12 does possess the advantage of binding more readily to protein than Orange G and thus the reaction with Acid Orange 12 takes less time to complete than with Orange G (Udy, 1971).

f Application of Dye-Binding to Determination of the Quantity of Crude Protein in Foods and Feedstuffs

Since the pioneer work of Frankel-Conrat and Cooper (1944), the dye-binding reaction has been extensively studied and used to determine the crude protein contents of various foods and feedstuffs ranging from dried skim milk powder to dry herbages. In the estimation of crude protein, one set of experimental conditions, such as the kind of dye used, time of mixing and protein:dye ratio, etc., established as optimal for a specific dye and a particular protein does not necessarily mean that those same conditions may be used

for another protein source. As a consequence, information in the literature on dye-binding procedures was abundant. In the review described below, data reported by various workers for the same protein source are discussed under the same heading. A few representative linear regression equations relating crude protein or nitrogen percent, as determined according to the Kjeldahl procedure, and dye-binding readings as reported by various workers are tabulated in Table 1.

1. Milk

Dye-binding with Orange G was first applied to milk protein determination by Udy (1956a). The procedure was later studied by Ashworth _et al_. (1960) who reported a correlation coefficient of 0.98 between protein contents determined by dye-binding and Kjeldahl procedures in studies involving 345 whole milk samples. In the same study, it was found that breed differences as well as butter fat and lactose contents of milk did not affect the results obtained. European workers (Schober and Hetzel, 1956), using the azo dye Amido Black 10B found that dye-protein binding was optimal at pH 1.9 and was not affected by the temperature of the milk sample. The suitability of Amido Black 10B for determining milk protein was later confirmed by Steinsholt (1957) and the procedure was further modified by Raadsveld (1958) to make the method more rapid.

Comparisons between Orange G and Amido Black 10B were reported by Ashworth and Chaudry (1962) and Dolby (1961).

Table 1

Linear Regression Equations Relating the Kjeldahl Protein Content and Dye-Binding Readings for Some Commonly Used Food and Feedstuffs

Protein material	Dye used	Regression equation[1]	Sample size	Corr. coeff.[2]	Reference
Milk	Orange G	$P= 5.60 - 3.05X$	345	0.98	Ashworth et al. (1960)
Milk	Acid Orange 12	$P=(1.25-C)/0.1824$			Sherbon and Fleming (1975)
Ground beef	Orange G	$P= 8.18 + 0.301B$	10	0.90	Torten and Whitaker (1964)
Ground pork	Orange G	$P= 5.43 + 0.367B$	11	0.80	Torten and Whitaker (1964)
Wheat	Orange G	$P= 44.47 - 40.00C$	128	0.992	Udy (1956a)
Wheat flour	Orange G	$P= 40.92 - 45.54C$	218	0.997	Udy (1956b)
Milled rice	Acid Orange 12	$P= 14.67 - 13.60X$	45	-0.961	Parial et al. (1970)
Brown rice	Acid Orange 12	$P= 14.78 - 14.12X$	45	-0.986	Parial et al. (1970)
Soybean meal	Orange G	$P= 0.945B - 23.1$		0.961	Moran et al. (1963)
Sesame flour	Acid Orange 12	$N= 0.3048 + 0.4358B$	14	0.991	Medina et al. (1976)
Rapeseed meal	Acid Orange 12	$N=- 0.8896 + 0.476B$	12	0.995	Medina et al. (1976)
Rapeseed flour	Acid Orange 12	$N=- 1.7337 + 0.4774B$	17	0.977	Medina et al. (1976)

---

[1] P, protein percent; X, absorbance of unbound dye in the supernatant; C, bound dye concentration; B, mg of dye bound per gram sample; N, mg of nitrogen as measured by Kjeldahl analysis.

[2] Correlation coefficient between the Kjeldahl protein contents and dye-binding readings.

Both groups of workers found that the two dyes were equally effective for the determination of milk protein. Both dyes gave linear relationships between protein and dye bound but Amido Black 10B had an advantage over Orange G in that it gave a much more sensitive optical indication of protein content. The protein values of milk as determined by dye-binding were not affected by refrigerated storage or pasteurization of the sample. The procedure of dye-binding for milk protein estimation has been investigated in collaborative studies and found to be highly reproducible within laboratories and acceptable between laboratories (Luke, 1967; Sherbon, 1967).

The high reproducibility and favourable acceptance of the dye-binding method by the dairy industry has stimulated work on automation of the procedure. The semi-automated Udy Protein Analyzer using Acid Orange 12 has been used experimentally as early as 1962 (Banasik and Gilles, 1962) and the Prometer Milk Analyzer using Amido Black 10 B has been produced in Europe (O'Connell , 1970). Both instruments have been tested for accuracy against the Kjeldahl procedure and found to be highly accurate and were recommended for official use by the Association of Official Analytical Chemists (Sherbon, 1967); Sherbon, 1975; Sherbon and Fleming, 1975).

A new azo dye Napthal Red S was recently tested by Konrad (1973a,b) and found to be suitable for milk protein determination. The correlation coefficient between the protein contents estimated by dye-binding and by the Kjeldahl

method for 15 samples was 0.98.

2. Meat

Torten and Whitaker (1964) were the first to study the use of Orange G and Amido Black 10B for estimating the protein contents of meats, including ground beef, pork, chicken breast and cod. When these foods were analyzed by the Orange G dye-binding method, the dye-binding capacity (DBC) per g Kjeldahl protein was found to vary with the protein content of the samples but the precision was poor. Neither Amido Black 10B nor Orange G was found to be suitable for this purpose. In both cases, there was evidence of a decrease in proportionate DBC of the protein with increase in protein content of the sample. Moss and Kiefsmeier (1967) studied various dye:protein ratios to find the optimal test condition for bindings. They favoured the use of Amido Black 10B and recommended a dye:protein ratio of 0.521 with a dye concentration of 0.105% for binding with protein in meat. The correlation coefficient obtained between DBC and protein content of the samples was, however, low ($r = 0.625$).

Ashworth (1971) re-investigated the use of pure Acid Orange 12 in the protein determination of meat products. He suggested that a range of dye:protein ratio from 0.64 to 0.92 was suitable for measurement of protein content since a calibration curve relating free dye concentration in the filtrate to protein content by the Kjeldahl method can be readily prepared for each type of meat product based on such ratios. The DBC of meat proteins was not affected by normal

cooking or fat content. Riqueline and Ashworth (1973) used the same dye (Acid Orange 12) on 90 samples of lamb meat and obtained a high correlation between percent protein determined by the dye-binding technique and the Kjeldahl method ($r = 0.99$) confirming the earlier findings of Ashworth (1971). The accuracy of values obtained by dye-binding was comparable to that obtained by the Kjeldahl method but reproducibility between duplicates was greater by dye-binding than by the Kjeldahl method. Since the dye-binding procedure appeared promising, an interlaboratory study involving four laboratories was initiated to evaluate the application of the Ashworth (1971) procedure in meat protein determination by Heller and Sherbon (1976). Using six samples of meat, they found that the correlations between the protein values determined by the two procedures at individual laboratories were all highly significant but they also found significant interlaboratory differences in the protein values estimated. Further studies are therefore required before application of the method is adopted.

3. Cereals

During the period when dye-binding was being studied, modified and automated for its use in milk protein determination, impressive achievements were also made in the application of the dye-binding principle to the estimation of the protein content of cereal grain.

Udy (1954) used Orange G in a study of four protein fractions isolated from wheat flour. He found that the

basic binding groups exhibited considerable constancy in their DBC within a given fraction for all varieties of wheat examined.

High correlation coefficients (0.992 and 0.997) between the concentration of unbound Orange G, which indirectly measured DBC, and the Kjeldahl protein content for 128 samples of wheat and 218 samples of wheat flour were reported by Udy (1956b). Mossberg (1965) using Amido Black 10B to determine the crude protein of wheat obtained lower protein values than estimated by the Kjeldahl method. Gruener *et al*. (1968) compared the use of Amido Black 10B versus Orange G for estimating protein content of wheat flour and favoured the latter.

Banasik and Gilles (1962) studied the precision of the Udy method for wheat protein analysis. Initial tests with the Udy Protein Analyzer appeared to indicate that the Udy Protein Analyzer was consistently giving low values in the high protein range and over-estimating protein contents in the low protein range. However, the values were corrected by a new conversion table obtained by recalibrating a standard curve covering a wide range of wheat protein contents. Greenaway (1972) also noted discrepancies in protein percent determined by dye-binding and the Kjeldahl procedure for 367 samples of wheat. Most mean differences were small, being less than 0.5% between dye-binding and Kjeldahl values except for wheats containing less than 10% protein where the mean differences approached 1%. Greenaway (1972) developed a

quadratic equation from the data he obtained from the Udy Protein Analyzer and Kjeldahl method and prepared a conversion table. The values obtained from the conversion table were in close agreement with Kjeldahl values.

Determination of the crude protein of other cereal grains by dye-binding was also reported by Mossberg (1968). Correlations between the dye-binding of five varieties of cereal grains and their nitrogen levels as analysed by the Kjeldahl method were; 0.97, 0.95, 0.96, 0.96 and 0.93 for barley, oats, wheat, rye and ryewheat respectively. The correlations between the amounts of dye bound and the total basic amino acids in the protein of the grains were equally high.

Olson and Heiges (1962) applied the dye-binding technique to routine barley protein analysis. Comparisons of the protein contents using the Kjeldahl and dye-binding methods on 577 barley samples indicated sufficient agreement to justify the use of the dye-binding method in routine barley protein analysis. The correlation coefficients for the different varieties of barley were all in excess of 0.96. Munck _et_ _al_. (1969) adopted this technique as a rapid screening process to determine the crude protein content of barley. The dye-binding method has been approved as an official AACC (American Association of Cereal Chemists) method (1970).

Determination of protein level in rice by the Udy Protein Analyzer was reported by Parial _et_ _al_. (1970). Mean protein contents of milled and brown rice estimated by the dye-binding method were not statistically different from those obtained by the Kjeldahl procedure. Kim _et_ _al_. (1971) also found high correlations between absorbance of the unbound dye and the protein content in brown rice.

4. Tubers

The dye-binding method has been used experimentally in the determination of protein in potato. Kaldy _et_ _al_. (1972) found a correlation of 0.98 between Kjeldahl protein and absorbance of bound dye by raw potato, using Orange G as the dye for protein binding. High correlations were also reported by Fritz and Munzett (1975) on air-dried or fresh potatoes. They indicated that Orange G is sufficiently accurate for routine determinations. Another dye, Bromophenol Blue (BPB) was used to estimate the 'true' protein of potatoes by Swaminathan _et_ _al_. (1973). Data collected from 120 samples of potatoes gave a correlation of 0.92 between the amount of BPB bound and the true protein content. Regression line relating the two variables passed through the origin, indicating absence of interference from non-protein nitrogenous substances. Protein values determined by both Orange G and BPB methods for dried potato samples were compared with their amino acid compositions by Desborough (1975). It was found that the values obtained by the BPB method were well correlated with those determined by amino acid analysis but the values

obtained by Udy Protein Analyzer were not as highly correlated with those determined by amino acid analysis as those obtained by the BPB method.

5. Forages

Quantitative estimation of protein in herbage by Orange G uptake was studied by Outen et al. (1966). Results obtained from study of 97 samples with 1 to 4% nitrogen content showed that although the uptake of Orange G increased proportionally as the nitrogen content increased, precision of the method was not as good as that of the Kjeldahl method. McKenzie (1976) recently compared the Orange G dye-binding method against the trichloroacetic acid (TCA) precipitated nitrogen for the determination of the protein content in dried pulp and leaf juice. It was found that the Orange G binding method was accurate and valid for estimation of TCA Kjeldahl nitrogen in leaf juice but less accurate for dried pulp unless separate regression equations were prepared for each species of dried pulp.

6. Oilseed Meals and Other High Protein Feedstuffs

One of the earlier studies on the use of Orange G binding as a measure of the protein content of high protein feedstuffs was presented by Bunyan (1959). Feedstuffs analysed included meat meals, whalemeat meals, fishmeals, soybean meals and groundnut meals. Linear regression equations were obtained for each of the individual protein sources, relating DBC and Kjeldahl protein contents. The

correlations for all the regression equations were highly significant except for meat meals in which a few atypical meals were encountered. Pomeranz (1965) also reported a high correlation between the protein values of finely ground soybean products determined by Orange G binding and by the Kjeldahl method (r = 0.98). However, the regression equation obtained was different from that reported by Bunyan (1959). Later, Hymowitz _et al_. (1969) modified the dye-binding determination by reading the protein content directly from the Udy Protein Analyzer and examined the relationship between values obtained by the Kjeldahl method and this modified version of the dye-binding technique. They found that the regression equation relating the protein values of 95 samples of soybean meal estimated by the two methods was linear in meals that ranged in protein content from 27 to 51%. The correlation coefficient was 0.985. The protein content as predicted by the modified dye-binding method accounted for only 94.3% of the total Kjeldahl protein. The difference was attributed to the presence of non-protein nitrogenous substances in the soybean meals which were measured by the Kjeldahl method but not by the modified dye-binding method. McCready _et al_. (1970) applied this technique to the determination of safflower meal protein but failed to find a relationship between dye-binding values and nitrogen contents as determined by the Kjeldahl procedure. In a study involving groundnut meals Daghir _et al_. (1969) reported that Orange G binding capacity was highly correlated with the crude protein content of five

groundnut meals (r = 0.98). Medina _et al._ (1976) using the Udy procedure obtained an equation for predicting the crude protein of sesame flour with a correlation coefficient of 0.991 from a sample size of 14.

Information on the application of the dye-binding method to RSM has become available only recently. Udy (1971) recommended an equation for the determination of the crude protein content of rapeseed by Acid Orange 12 binding. However, the validity of the equation was not supported by any experimental data. Medina _et al._ (1976) studied the application of the dye-binding technique to RSM. They modified the Udy procedure (Udy, 1971) by reducing the mixing time to 30 minutes and predicting the crude protein contents of RSMs and rapeseed flours by the amount of dye bound to one gram of sample. The DBC was then related to nitrogen content determined by the Kjeldahl method. The predicted protein values for 17 samples of rapeseed flour and 12 samples of RSM were not significantly different from the Kjeldahl protein values. Linear regression equations derived from rapeseed flour and meal are shown in Table 1 and correlations between the two methods for flour and meal were 0.977 and 0.995 respectively. The dye-binding method had better precision than the Kjeldahl method as shown by the lower standard deviation of 0.338 versus 0.595% protein for Kjeldahl method.

g Application of Dye-Binding to Determination of the Quality of Protein

Although the majority of reports cited have clearly demonstrated the validity of the dye-binding method in

quantitative determination of crude protein, none have demonstrated a relationship between values obtained and protein quality other than indicating that the values obtained give a good estimate of the total basic amino acids in the sample analysed.

Careful examination of the principle involved in the dye-protein reaction, however, revealed that the dye-binding procedure has another advantage over Kjeldahl nitrogen determination in that it is capable of detecting changes which might alter the contents of the three basic amino acids, particularly the essential amino acid lysine. It is well known that when a protein material is subjected to excessive heat treatment the heat-labile and often limiting amino acid lysine is chemically altered in such a way that the epsilon amino group in its molecule becomes bound to carbohydrate and the nutritive value of the protein is greatly reduced (Carpenter and Booth, 1973). In this instance, the nitrogen content by the Kjeldahl method remains the same but, not so by the dye-binding procedure. The amount of dye bound to the heat-damaged protein will be reduced in proportion to the amount of lysine destroyed. Thus, it appears that combined analysis for nitrogen by the Kjeldahl and dye-binding methods could be used to measure the protein quality of a protein-rich material.

1. Detection of Heat Damage to Protein

Changes in the DBC of a protein containing sample appear to be largely dependent on the severity of the heat applied.

Mild heat treatment of protein over a short period of time does not seriously affect the content of amino acids and as a result the DBC of the protein. Egg albumen samples denatured by heating for 5 minutes at 70°C and pH 2.5 and 11.7 did not affect the DBC of the albumen samples according to Frankel-Conrat and Cooper (1944). Neither were adverse effects noted in liquid milk subjected to heating at temperatures as high as 190°F (Vanderzant and Tennison, 1961). Sterilization heat, intense enough to cause browning of the milk did, however, lower the DBC of the milk protein (Tarassuk, 1967). During his study of the application of dye-binding on milk protein determination, Udy (1956a) demonstrated that liquid whole milk and dried milk powder bound with different amounts of Orange G per g protein and attributed this difference to the denaturation of the milk protein during the drying process.

Mossberg (1965) studied the DBC of wheat and demonstrated a noticeable effect of heat treatment on the ability of wheat protein to bind Orange G. Ground wheat samples heated at 100°C for 16 hour showed consistently lower protein values than those recorded for the corresponding unheated samples when determined by the dye-binding method. Additive effect of temperature, water content and duration of heating on the uptake of dye by cereal grain was also examined by Mossberg (1966). Ground barley samples with moisture contents of 12.0, 21.1 and 27.9% were heated at 50° and 85°C from 8 to 96 hours. The protein contents according to Kjeldahl analysis did not change and were independent of temperature, water

content and the duration of heat treatment. On the contrary, the protein contents by dye-binding estimation changed after heat treatment as a function of temperature, moisture and duration of heating.

Influence of heat treatment on the DBC of oilseed meals has been studied mainly with soybean meal. Moran et al. (1963) studied the binding of Orange G by heated soybean meals and found that 15 minutes of heat treatment significantly reduced the DBC and further reduction occurred when the autoclaving was prolonged to 1 and 2 hours. Using the ratio of DBC of the unheated and heated meals as index of quality, they found that the protein quality of soybean meals heated for 0, 45 and 90 minutes were significantly different from each other, a result which was confirmed by biological assay. Application by these workers of the technique to fishmeal was not successful because of the fluctuations in histidine and arginine contents among species or parts of fish used in fishmeal production. Similar reduction in DBC of soybean flour subjected to autoclaving was reported by Pomeranz (1965) and of soybean meal by Hymowitz et al. (1969) and in meat and bone meals by Choppe and Kratzer (1963). In all of these studies the samples were subjected to rather severe heating before a decrease in DBC was noted.

The various stages of heat destruction to food protein were studied in detail under simulated conditions using isolated protein by Hurrell and Carpenter (1975). The degree of protein damage was measured by the dye-binding procedure

with three different dyes, a reactive dye, Remazol Brilliant Blue, a phthalein dye, Cresol Red, and an azo dye, Acid Orange 12. Their findings could be summarized as follows: for animal feedstuffs unheated or deliberately heated, the Acid Orange 12 binding values were close to the sum of total histidine, arginine and lysine with free epsilon amino groups. Both the dye-binding values and the sum of basic amino acids were reduced similarly by heat treatment. However, for protein materials subjected to early 'Maillard' reaction, dye-binding values with Acid Orange 12 were unchanged even though the total content of basic amino acids was considerably reduced. Bindings of the other two dyes were found to be unsatisfactory.

2. Selection of High Lysine Variety of Cereal Grains

Another important application of the dye-binding technique is in the selection of high lysine varieties of cereal grains in plant breeding work. The basis of the application is that, of the three basic amino acids in cereal grains, lysine is often the only limiting one and varies among varieties, whereas, the levels of histidine and arginine remain relatively constant. Hence, two samples of the same cereal grain containing identical amounts of protein according to Kjeldahl analysis but which bind different amounts of dye would probably contain different levels of lysine.

A new line of hybrid corn known to contain higher lysine than the original line was successfully identified by this method (Mossberg, 1966). The two lines, analysed by the

Kjeldahl procedure to contain 13.26 and 13.29% crude protein, bound 247 and 336 mg of dye per 100 g protein respectively. Of 67 samples from six varieties of cereals, the correlation between lysine levels determined by an amino acid analyzer and DBC was higher than that between lysine and the nitrogen contents (r = 0.94 versus 0.80). This observation lends further support to the use of DBC as a suitable screening technique for lysine provided there is a good positive correlation between the total basic amino acids and lysine for the particular type of cereal studied. This procedure has been adopted by Munck _et al_. (1969) in his selection for a high protein, high lysine variety of barley by screening 2500 varieties of barley from the world collection.

Bhatty and Wu (1975) have recently presented a modified dye-binding procedure for screening barley samples for lysine content. Using a sample weight and dye concentration (Acid Orange 12) one-third of those used in the Udy (1971) method, a standard conversion table relating the transmittance reading to the lysine content of barley was prepared. Lysine levels of 34 lines of barley estimated by this modified dye-binding procedure were highly correlated to values obtained by an amino acid analyzer for the same samples. The advantage and reliability of this modified procedure was later confirmed by Laberge _et al_. (1976).

Juliano _et al_. (1973) used a similar procedure to screen for a high lysine variety of rice. Of more than ten thousands varieties examined from the world collection, only 38

varieties were found to contain higher total basic amino acids of which seven of them with high DBC had 0.5% higher lysine content than the mean value for brown rice.

h Correlations Between Protein Qualities of Feedstuffs as Predicted by the Dye-Binding Method and by Biological Assays

As indicated previously in this review, the dye-binding method used in conjunction with Kjeldahl nitrogen analysis, is a useful laboratory means for detecting inferior protein quality caused by excessive heat treatment. However, its practical applicability can be justified only if the predicted protein quality of feedstuff is highly correlated with results derived from biological assays.

As early as 1957, the DBC of fishmeals with Orange G was studied for its correlation with their nutritive values (Thurston, 1957). Orange G absorption test was one of the methods used in predicting the protein quality of a wide range of protein foods by Bunyan and Price (1960). The same samples were also assayed biologically by the net protein utilization (NPU) method with rats. By comparing the Orange G binding data with the NPU values, it was found that the Orange G dye absorption correlated well with the NPU values when expressed as acid equivalents per 10^4 g of protein. The whalemeat meals, meat meals and fishmeals that had higher dye-absorption values had higher NPU values. This was true also for the numerous other protein feedstuffs.

Similar agreement between dye-binding and biological assays was reported by Boyne _et al_. (1961) for numerous protein

concentrates from both plant and animal origins. Of the different kinds of oilseed meals studied, only the groundnut meal samples failed to show a correlation between the dye absorption values and the corresponding gross protein value (GPV) results recorded in chick assays.

Studies on meat and bone meals were reported by Choppe and Kratzer (1963). Twenty samples of meat and bone meal were used in chick growth assays as the sole source of protein. The meals were used to supply 24% of protein in a purified type ration and fed to chicks from the fifth to the fifteenth days of age. The amounts of Orange G bound to the meat and bone meals were poorly but positively correlated to chick growth data (r = 0.64).

Moran _et_ _al_. (1963) studied the effect of heat treatment on the DBC of soybean meals and verified the results by chick assays. The meals autoclaved for periods ranging from 15 minutes to 2 hours were included at the 35% level in rations fed to chicks for four weeks. The decline in protein quality as measured by the chicks weight gain corresponded to the protein quality indices predicted chemically by DBC of the meals tested with the exception of the raw, unheated meal. Although the unheated soybean meal showed optimal binding with Orange G, it supported poorer growth than meal heated for 15 minutes. This was attributed to the presence of certain anti-nutritional factors in soybean meal. Both weight gain and dye-binding indices showed that the qualities of the meals were significantly reduced over that found after 15 minutes of autoclaving only after autoclaving exceeded 60 to 90 minutes.

Comparative assessment of the protein qualities of three fishmeals by the Orange G binding test and by chick growth assays were reported by Bunyan and Woodham (1964). The chick growth was measured by protein efficiency ratio (PER). The protein quality of the meals as predicted by Orange G dye uptake was closely correlated with the values obtained in chick assays.

Study of cereal grains was presented by Munck (1966). Several barley samples of known DBC were evaluated for their protein quality by weight gain to 20 days of age in mice feeding tests. A clear correlation between DBC of the barley samples and weight gain was demonstrated.

A recent study which showed close correlation between protein qualities predicted chemically by dye-binding and biologically by chick growth and NPU test in rats was reported by Carpenter and Opstvedt (1976). Eight samples of fishmeal were evaluated by laboratory methods and tested for their potency as sources of lysine for animal growth. When the mean results obtained from the various tests were compared with bioassay, the dye-binding test was found to be highly correlated with the chick growth and NPU values. The correlation coefficients found were 0.933 between dye-binding and chick assay and 0.742 for NPU assay.

i Determination of Nutritionally Available Lysine Content of Feedstuffs by Differential Dye-Binding Capacity

The term 'available lysine' refers specifically to the amino acid lysine with a free epsilon-amino group and is nutritionally available to the animal upon ingestion of

the protein material. It was introduced mainly to distinguish the 'available' form from the total lysine which is conventionally estimated by an amino acid analyzer and includes the 'available' as well as the unavailable lysine. At the time when correlation of amino acid composition of proteins and their nutritive values was being demonstrated, there was already some evidence that the reduced nutritive value of the protein of dried milk resulting from storage was related to the poor availability of lysine (Henry et al., 1948). This was later verified by study of a casein-glucose model but also found true for other protein sources (cited by Carpenter and Booth, 1973).

Chemical methods developed for measuring available lysine content of feedstuffs were based on the reaction of the free epsilon-amino group of lysine with 1-fluoro-2, 4 dinitrobenzene (FDNB), a chemical used by Sanger (1945) for identifying the number of different N-terminal groups in proteins and peptides. The amount of available lysine was measured by quantitative determination of ξ-dinitrophenyl lysine (DNP-lysine) formed. The analytical procedure has been outlined by Carpenter (1960) and later modified by Booth (1971). Various modifications of the Carpenter (1960) procedure have been suggested for isolation of the DNP-lysine prior to spectrophotometric determination of the compound (Blom et al., 1967; Matheson, 1968; Holm, 1971). Roach et al. (1967), on the other hand, estimated the available lysine content by measuring the difference between the total lysine and unavailable lysine

content, that is the free lysine which was released on acid hydrolysis after the reaction with FDNB. Ostrowski et al. (1970) introduced slight changes in chromatographic conditions to improve the resolution of lysine and ornithine. Despite all of the improvements and modifications proposed for the analytical procedure, none of them is at present, considered faultless.

The dye-binding technique has recently been studied for its application in determining 'available lysine' content of protein commodities. This has led to the development of the new technique known as differential dye-binding capacity procedure. The determination is performed by measuring the amount of dye which binds with the protein before and after the free epsilon-amino group of lysine was masked by a chemical compound. The difference in DBC per unit weight of protein for the two determinations gives the equivalence of dye bound to the 'available lysine' in the protein. The modified method appears to be theoretically sound and if applied successfully would have the advantages of bypassing the cumbersome and lengthy acid hydrolysis and DNP-lysine isolation required in other methods.

Sandler and Warren (1974) were the first to put this hypothesis to test. For the selection of the reagent to mask effectively the free epsilon-amino groups of lysine, they suggested that a compound selected for such purpose should be of small molecular size to avoid steric hindrance to reaction of dye with the basic groups. Other than that, it

should react quantitatively with the epsilon-amino group without altering the basic properties of the imidazole group of histidine and guanidine group of arginine. Using ethyl chloroformate as the blocking agent, Sandler and Warren (1974) developed a practical analytical procedure for measuring the lysine content of fishmeal protein. Results obtained showed a close agreement between the differential equivalent DBC for fishmeal protein and lysine in the sample. The same procedure was found applicable to soybean meal but not suitable to gelatins.

Based on the same theoretical consideration, Jones and Lakin (1976) followed a similar procedure to estimate the lysine level of barley samples by determining the dye-binding difference (DBD) before and after blocking the epsilon-amino group of lysine with trinitrobenzene sulphonic acid (TNBS) at 40°C. A total of 24 feed barley samples were analysed. There was a strong correlation ($r = 0.951$) between the DBD values and their lysine levels as analysed by an amino acid analyzer. Hurrell and Carpenter (1976) used propionic acid to mask the free epsilon-amino group and measured the dye-binding capacity of samples before and after propionylation. The DBD values of the samples agreed with the available lysine levels determined by the Carpenter (1960) procedure for cereal grains, groundnut flour and soybean meal but fishmeal and meat meals usually gave lower DBD values than those obtained by the FDNB method. However, it should be noted that the differential dye-binding technique is still in

a rather early stage of development. With further refinement of the experimental conditions and selection of a suitable chemical for masking the free epsilon-amino group of lysine, the method might prove to be an indispensable tool for rapid estimation of the biologically 'available lysine' in feedstuffs.

C Experiments Conducted at The University of Alberta

a Experiment 1: Study of the Correlations Between the Crude Protein Contents and the Dye-Binding Capacities of Rapeseed Meals with Orange G and Acid Orange 12

1 Objective

The first of this series of experiments was designed to study the relationships between the crude protein contents as estimated by the Kjeldahl method and the dye-binding capacities (DBCs) of rapeseed meals (RSMs) with two dyes commonly used in dye-binding studies, namely, Orange G and Acid Orange 12.

2 Materials and Methods

Fifteen samples of RSM were assembled for the study. The samples were known to differ widely in protein contents. Thirteen of the samples were obtained from commercial rapeseed processing plants while two of the meals were produced in the laboratory. Prior to the study of DBC, the nitrogen contents of the samples were determined by the Kjeldahl procedure (A.O.A.C., 1970) and the percentages of crude protein were calculted using the conversion factor of 6.25. The moisture contents of the RSMs were determined by drying in a vacuum oven (A.O.A.C., 1970).

In connection with the determination of the contents of basic amino acids in the RSMs, 1 g samples of the meals were placed in 250 ml of flat bottom flasks fitted with a water cooled condenser and 25 ml of 6N HCl was added to each flask. The mixture was refluxed for 24 hours. Excess HCl was re-

moved under vacuum using a rotary evaporator. The residue was washed twice with distilled water, each time being evaporated to dryness in a rotary evaporator. The residue was made to 100 ml in a volumetric flask with de-ionized distilled water. A portion was filtered through a millipore filter attached to a syringe and the filtrate was analysed for basic amino acids using a JLC-5AH Amino Acid Analyzer (JEOL Co., Japan).

In an attempt to establish the experimental conditions required for optimal dye-protein binding, the effects of particle size and of protein:dye ratio in the mixture on the DBC of RSM with Orange G were studied. Only the effect of particle size on the DBC with Acid Orange 12 was investigated.

Particle size. Four samples of RSM were ground in a laboratory microanalytical mill to sizes which passed through 40 (0.42 mm) and 60 (0.25 mm) mesh sieves, respectively, and their uptakes of Orange G were studied by the procedure outlined by Moran *et al*. (1963). Results obtained are shown in Table 2. Statistical analysis of the data by Student's t test revealed no significant difference between the mean DBC of the 40 mesh versus the 60 mesh particle size ($P<0.05$).

Seven samples of RSM were ground to the particle sizes referred to above and were studied for their DBCs with Acid Orange 12 using the Udy Protein Analyzer (Udy, 1971). The results obtained showed no significant difference in the amounts of Acid Orange 12 bound by one gram samples ground to 40 or 60 mesh size (Table 3). A particle size of 40 mesh was therefore selected.

Table 2

Effect of Particle Size of Sample on the Amount of Orange G Bound by Rapeseed Meal

Sample	mg Orange G bound per gram of meal	
	40 mesh[1]	60 mesh
1	72.6	74.2
2	66.3	66.7
3	62.8	63.3
4	62.2	64.0
Mean[2]	66.0	67.0

[1] 40 mesh = 0.42 mm, 60 mesh = 0.25 mm.

[2] Means for DBC at 40 and 60 mesh were not significantly different ($P<0.05$).

Table 3

Effect of Particle Size of Sample on the Amount of Acid Orange 12 Bound by Rapeseed Meal

Sample	mg Acid Orange 12 bound per gram of meal 40 mesh[1]	 60 mesh
1	139.8	141.2
2	137.4	138.5
3	126.6	125.3
4	124.1	124.2
5	128.7	125.5
6	129.1	129.8
7	123.7	121.3
Mean[2]	129.9	129.4

[1] 40 mesh = 0.42 mm, 60 mesh = 0.25 mm.

[2] Means for DBC at 40 and 60 mesh were not significantly different ($P<0.05$).

Protein:dye ratio. Seven samples of RSM were studied for their DBCs with Orange G by the procedure of Moran *et al.* (1963). Protein:dye ratios of approximately 4:1 and 2:1 were tested for optimal binding of RSM protein with Orange G. The ratios were obtained by using 250 and 150 mg samples of RSM to react respectively with 25 ml of 0.1% Orange G solution. The results (Table 4) clearly indicated that the mean amount of Orange G bound per g of meal was significantly higher for the protein:dye ratio of 2:1 than for the ratio of 4:1. Although at both ratios, the DBC measured was highly correlated with the Kjeldahl protein levels of the meals, the ratio of 2:1 was chosen to ensure a more complete dye-protein reaction.

Dye-Binding Capacity of Rapeseed Meal with Orange G

150 mg of RSM ground to 40 mesh, was transferred to a 60 ml polyethylene flask and 25 ml of 0.1% Orange G dye solution was delivered to the flask. (The 0.1% Orange G solution was prepared by dissolving 1 g of recrystallized Orange G in phosphate buffer solution of pH 2.2 which contained 980 ml of 0.1 M citric acid and 20 ml of 0.2 M disodium phosphate). The RSM-dye mixture was then placed on a shaker and shaken at the rate of 120 strokes per minute for 1 hour. It was then filtered through a filter cap fitted with a fibre glass filter paper and a 1 ml aliquot of the filtrate was diluted 1:100 in a volumetric flask with distilled water.

Optical density of the diluted filtrate was recorded colorimetrically at 470 nm in a Beckman DBG Spectrophotometer. The amount of unbound dye in the filtrate was obtained from

Table 4

Effect of Protein to Dye Ratio on the Amount of Orange G Bound by Rapeseed Meal

Sample	Kjeldahl protein (%) (N x 6.25)	mg Orange G bound per gram of meal Protein:dye ratio 4:1	 2:1
1	44.8[1]	66.1	80.9
2	40.4	62.6	73.3
3	39.8	62.4	72.6
4	36.5	56.9	66.3
5	36.6	56.0	62.8
6	36.8	57.8	62.2
7	33.9	54.1	58.7
Mean[2]		59.4	68.1

[1] Correlation coefficients between Kjeldahl protein and dye-binding capacity at protein:dye ratios of 4:1 and 2:1 were identical ($r = 0.98$).

[2] Means for DBC at 4:1 and 2:1 protein to dye ratios were significantly different ($P<0.05$).

a calibration curve prepared by determining the optical density of appropriately diluted stock dye solution over the range of 0 to 1.0 mg/ml at 0.1 intervals. Determination of the DBC of each RSM sample was done on five replicates. From the known amount of unbound dye and the sample weight, the DBC was calculated.

Dye-Binding Capacity of Rapeseed Meal with Acid Orange 12

A sample size of 250 mg of meal was used to react with 40 ml of Acid Orange 12 stock solution (1.3 mg/ml) for estimating the amount of Acid Orange 12 bound by RSM. (The stock solution was prepared by diluting Reagent Acid Orange 12 solution, Udy Co., Boulder, Colorado, with distilled water). This provided approximately 14 mg of protein nitrogen in the dye-protein mixture as suggested by Udy (1971). The DBC of the RSMs with Acid Orange 12 was determined according to the procedure of Udy (1971) by measuring the transmittance of the undiluted filtrate in a colorimeter equipped with a short light-path cuvette. The unbound dye concentration was obtained by referring the transmittance to the conversion chart provided. To allow cross-checking of the values obtained with Udy's colorimeter, the dye concentration of the same filtrate was also estimated by the spectrophotometric method. For this purpose, 1 ml of the filtrate was diluted to 100 ml with distilled water in a volumetric flask. The optical density of the diluted solution was read in a Beckman DBG Spectrophotometer at a wavelength of 480 nm. The concentration of the unbound Acid Orange 12 which remained after

removal of the dye-protein complex was read from a calibration curve relating the optical density and dye concentration prepared in a manner similar to that used in connection with determinations involving Orange G.

3 Results and Discussion

The crude protein, dry matter and basic amino acid contents of the 15 RSM samples studied are shown in Table 5. The total amount of basic amino acids per g of RSM was also computed and presented in the same table.

The crude protein contents of the meals (N x 6.25) analysed by the Kjeldahl method were found to cover a wide range (30.1 to 44.8%). This was considered desirable. Variation in lysine, arginine and histidine contents of the different meals were of the same order. The moisture contents of the samples were relatively constant.

The dye-binding data for the RSMs tested are summarized in Table 5. For both Orange G and Acid Orange 12, the amount of dye bound to one gram of RSM decreased as the level of protein decreased. The relationship between the DBC (mg Orange G/g rapeseed meal) and the percent protein obtained by the Kjeldahl method is shown in Fig. 2. The regression equation relating DBC (x) and protein percent (Y) was:

$$Y = 3.91 + 0.49x.$$

The correlation coefficient was 0.93 and the standard deviation was 1.38% of the protein. Uptake of Acid Orange 12 by the RSMs as determined colormetrically by Udy's colorimeter and spectrophotometrically were plotted against percent

Table 5

Crude Protein, Basic Amino Acids and Dry Matter Contents of Rapeseed Meals and Dye-Binding Capacities of Rapeseed Meals with Orange G and Acid Orange 12

Sample	Crude protein (N x 6.25)	Basic amino acid (g/16g N)			Total BAA mg/g meal	Dry matter %	Orange G bound mg/g meal	Acid Orange 12 bound mg/g meal	
		Lysine	Histidine	Arginine				Udy's method	Spectro-photometric method
1	44.8	6.07	2.84	6.46	68.9	92.0	80.9	157.4	157.2
2	40.4	5.94	2.76	5.92	59.2	91.7	73.0	144.0	142.1
3	40.3	5.75	2.66	5.94	57.8	92.1	73.3	139.5	139.8
4	39.8	5.86	2.82	6.19	59.2	91.4	72.6	138.4	137.4
5	37.5	5.60	2.71	6.46	55.3	91.4	71.7	131.5	131.8
6	36.5	5.64	2.74	5.57	50.9	91.0	66.3	126.6	126.6
7	36.6	6.30	2.77	5.77	53.6	90.7	62.8	122.5	124.1
8	36.8	6.10	2.79	5.79	54.0	90.3	62.2	127.1	128.7
9	36.6	6.27	2.79	5.94	53.2	93.1	62.3	129.7	129.1
10	36.4	5.77	2.79	5.94	52.8	91.9	67.1	124.4	124.8
11	36.0	5.82	2.70	6.13	52.8	92.2	66.1	120.6	121.0
12	34.6	5.93	2.75	6.03	50.9	92.0	65.3	123.2	123.4
13	33.9	6.86	2.80	6.32	54.2	91.7	58.7	123.5	123.7
14	32.6	5.74	2.61	5.88	46.4	90.3	58.7	114.5	115.5
15	30.1	5.84	2.75	5.99	43.9	92.4	57.4	103.7	104.9
r[1] value with crude protein	1.00						0.93	0.98	0.98
r value with total BAA					1.00		0.87	0.96	0.97

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[1] All 'r' (correlation coefficient) values were highly significant (P<0.01).

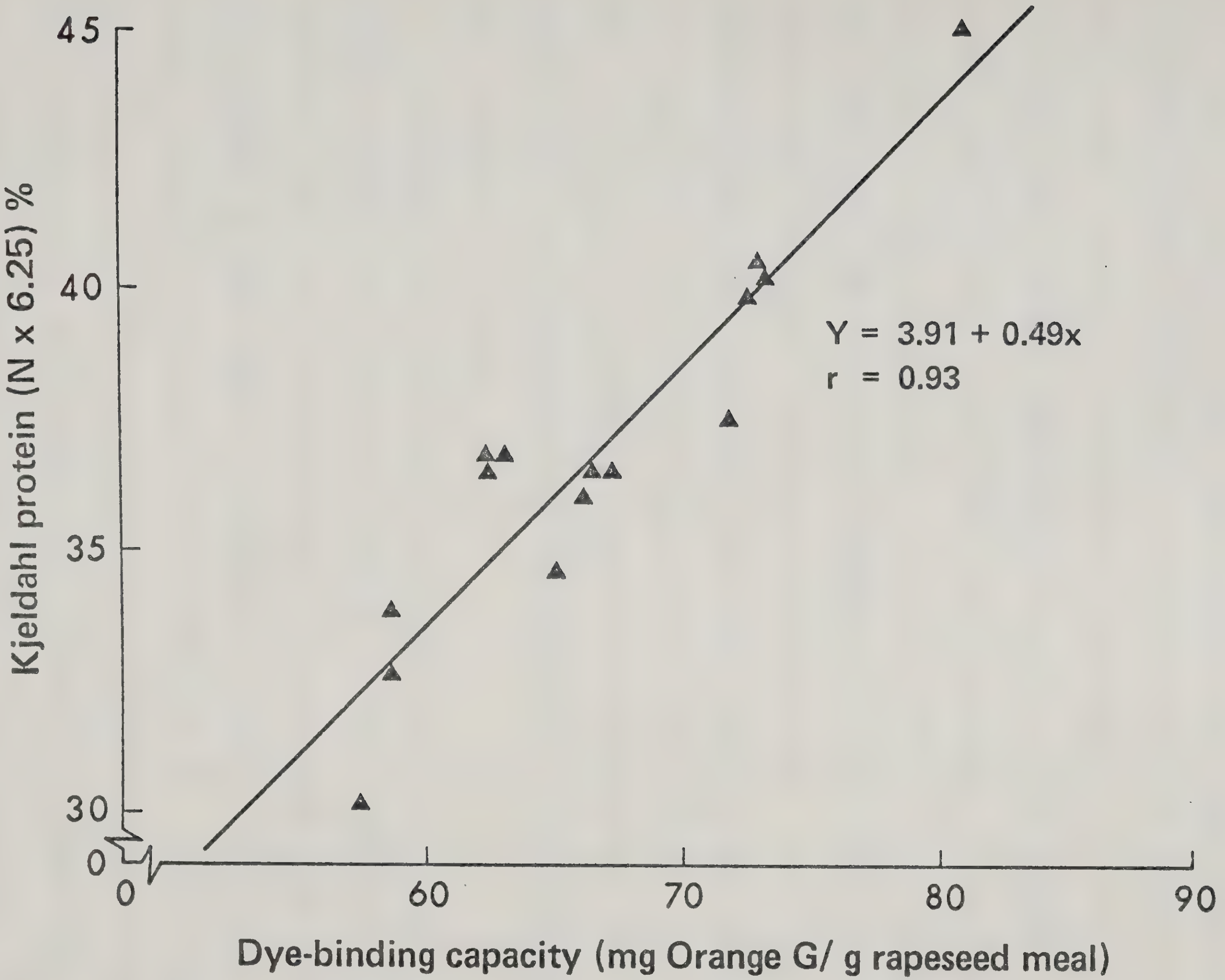

Fig. 2 The relationship between the dye-binding capacities of the the rapeseed meals with Orange G and the Kjeldahl protein contents of the meals.

protein and are shown in Fig. 3. Regression equations showing linear relationships between Acid Orange 12 binding (x) and Kjeldahl protein percent (Y) were: $Y = 2.20 + 0.27x$ and $Y = 0.36 + 0.28x$ for the colorimetric and spectrophotometric determinations respectively. The close relationship between the protein level in the RSM and the amount of Acid Orange 12 bound was clearly demonstrated by the high positive correlation coefficients (0.98 for both) obtained. The standard deviations calculated were 0.80 and 0.79% of the protein. The same data were further analysed statistically by quadratic regression analysis (Steel and Torrie, 1960) but there appeared to be no advantage for this analysis at the 5% level as compared to the linear regression. Therefore, it seems that the linear regression equations obtained for the two dyes tested are adequate for satisfactory estimation of protein contents of RSMs.

Although for both dyes, the amount of dye bound per unit weight of RSM was linearly related to the crude protein of the meal, the use of Acid Orange 12 seemed superior to Orange G as shown by the higher correlation coefficients and the lower standard deviation for Acid Orange 12. It is also evident that the DBC as determined by direct reading of undiluted filtrate in the Udy colorimeter, is comparable to that measured by the spectrophotometer and suggests the former as the method of preference.

The fact that all the linear equations do not pass through the origin suggests that some interference with the dye-protein

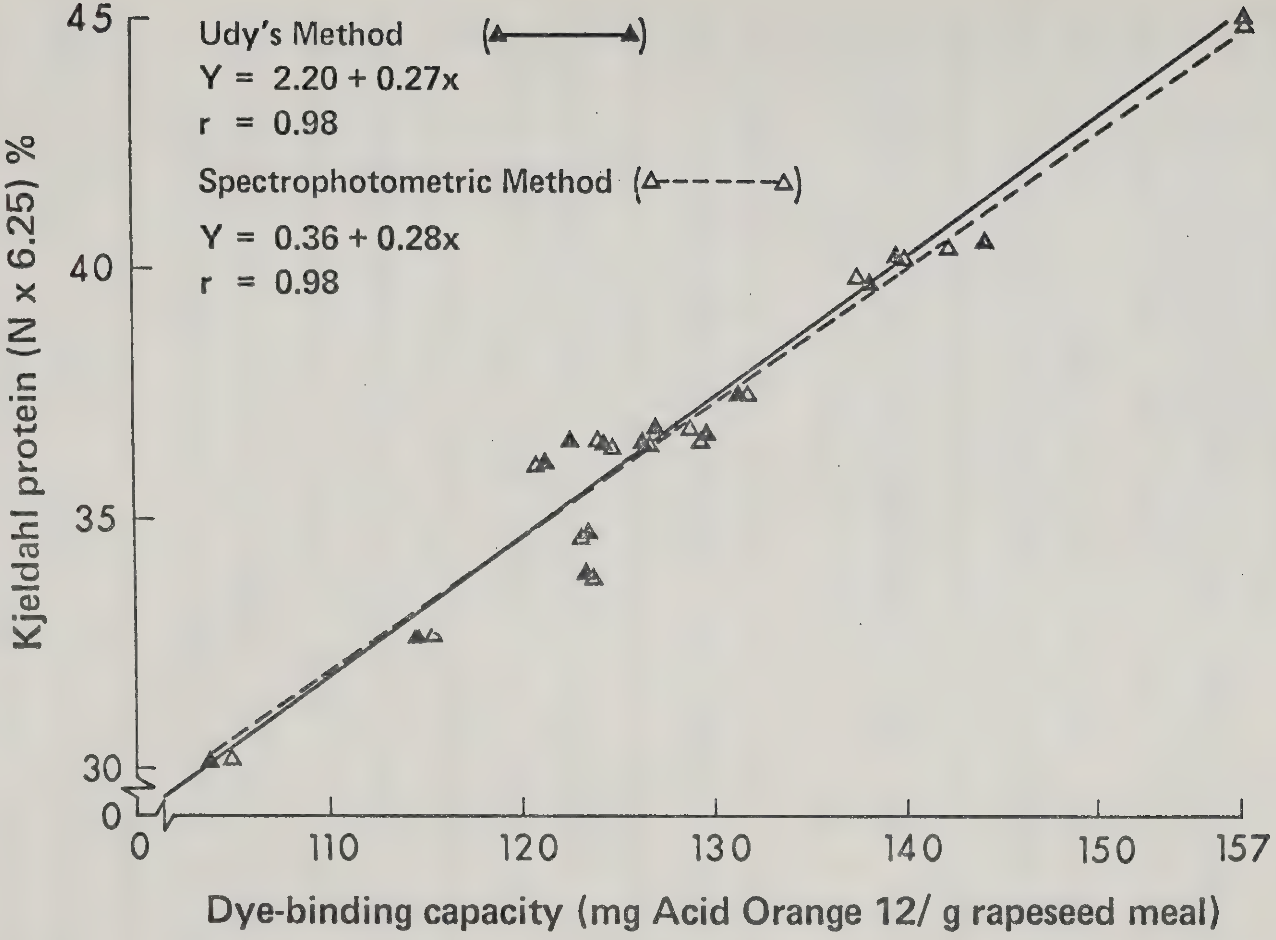

Fig. 3 The relationship between the dye-binding capacities of the rapeseed meals with Acid Orange 12 as determined by Udy and spectrophotometric methods and the Kjeldahl protein contents of the meals.

reaction by other non-protein substances was taking place. Ideally, the line represented by the linear equation for predicting protein content should pass through the origin with a correlation coefficient of 1.

Since the azosulphonic dyes used bind mainly to the basic amino acids, the absolute amount of the basic amino acids (BAA) in each meal was calculated and expressed in mg BAA/g rapeseed meal (Table 5). The correlation coefficients between DBC and BAA contents were 0.87 for binding with Orange G measured by the spectrophotometer and 0.96 and 0.97 for binding with Acid Orange 12 measured by Udy colorimeter and the spectrophotometer respectively. These results agree well with the earlier findings and support the use of Acid Orange 12 as a more suitable dye for estimation of the crude protein of RSM. A possible explanation for the less satisfactory results with Orange G as compared to Acid Orange 12 is that physical blocking of the formation of the protein-dye complex might arise with Orange G due to the presence of two binding sites per Orange G molecule. Steric hindrance may prevent the complete binding of these sites.

Results recorded in this study compare favourably with values reported for other oilseed meals (Hymowitz et al., 1969; Daghir et al., 1969). However, the correlation coefficients obtained were slightly lower than those reported by Medina et al. (1976). These workers reported correlation coefficients of 0.977 and 0.995 between the nitrogen content and the amount of Acid Orange 12 bound by one gram of rapeseed flour and RSM

respectively. The correlation coefficient of 0.995 obtained for RSM in their study was based on measurements made on 12 samples of RSM which varied little in their protein contents since they were drawn from a single lot of RSM. In the present study, the RSM samples used in deriving the correlations between DBC and protein content covered a wide range of protein contents.

The practical application of the regression equation obtained for Acid Orange 12 using the Udy method will be investigated in the next experiment for mass screening the crude protein of commercial RSMs.

4 Summary

Fifteen RSMs were used to study the relationships between the Kjeldahl protein contents of the meals and their dye-binding capacities (DBCs) with the two dyes, Orange G and Acid Orange 12. The DBCs of RSM with Orange G were determined by a spectrophotometric method and the DBCs of the meals with Acid Orange 12 were measured by an Udy colorimeter and by a spectrophotometer. Correlations between DBC and percent Kjeldahl protein and between DBC and total basic amino acids in the meals were found to be highly significant. The results obtained favoured the use of the Udy method and Acid Orange 12 as the dye.

b Experiment 2: Estimation of the Crude Protein Contents of Commercially Processed Rapeseed Meals by Dye-Binding Capacity with Acid Orange 12

1 Objective

This experiment was a follow-up of the previous experiment and was designed to test the applicability of the linear regression equation established in Experiment 1 for the estimation of the crude protein contents of commercial rapeseed meals (RSMs). The equation ($Y = 2.2 + 0.27x$) selected related the crude protein percent and the dye-binding capacity (DBC) of RSM as determined by the Udy's method.

2 Materials and Methods

A total of 126 samples of RSM were collected for the study. Most of the samples were obtained from four major processing plants. The varieties of the seeds from which the meals were produced were not known. The RSMs were numbered and reground in a cyclone mill to reduce the particle size to 40 mesh.

The RSMs were analysed for crude protein contents by the macro-Kjeldahl method (A.O.A.C., 1970) and their DBCs were determined by Udy's method (Udy, 1971) with Acid Orange 12. In addition, certain selected samples were further analysed for basic amino acids as described in Experiment 1 and for available lysine according to the procedure of Booth (1971).

3 Results and Discussion

The data obtained on the 126 RSMs, are summarized in Table 6. The crude protein contents of the meals varied from 32.4 to 39.0% and more than 90 of the samples were within the range of 34-37% protein. The DBCs recorded for the samples were also plotted against their crude protein percents and are shown in Fig. 4. A linear relationship between the two variables was seen but some degree of scattering was noted. The correlation coefficient between the two variables was 0.78. Although the value was highly significant ($P<0.01$), it was unexpectedly lower than the 'r' of 0.98 obtained from the 15 RSMs used in the preparation of the calibration curve in Experiment 1.

The predicted protein values based on their DBCs by the theoretical equation $Y = 2.2 + 0.27x$ where Y is the predicted protein percent and x, the DBC, are shown in Table 6. From the differences calculated between the analysed Kjeldahl protein percents (N x 6.25) and the predicted protein percents, it appears that the predicted values over-estimated the Kjeldahl protein contents of meals when the N x 6.25 was less than 35% and under-estimated the protein contents of the meals when it exceeded 35%. The frequency distribution of the differences in protein percent are shown in Table 7. From the frequency distribution shown, it will be noted that approximately 80% of the predicted values fell within ±1% of the observed values and about 60% of them were within ±0.6%.

Table 6

Differences Between Observed Crude Protein and Predicted Crude Protein Percents of Rapeseed Meals Based on Their Dye-Binding Capacities

Sample[1]	Observed crude protein (N x 6.25) a	DBC[2] mg/g meal	Predicted crude protein b	Difference b-a	DBCP[3] mg/g protein
1*	32.4	117.1	33.8	1.4	361.4
2	32.9	116.8	33.7	0.8	355.0
3*	33.2	118.0	34.1	0.9	344.0
4	33.4	120.4	34.7	1.3	360.5
5*	34.0	124.2	35.7	1.7	365.3
6	34.1	120.2	34.7	0.6	352.5
7	34.3	120.3	34.7	0.4	350.7
8	34.3	120.8	34.8	0.5	352.2
9	34.3	122.6	35.3	1.0	357.4
10	34.4	121.3	35.0	0.6	352.6
11	34.5	121.0	34.9	0.4	350.7
12	34.5	120.0	34.6	0.1	347.8
13	34.5	118.9	34.3	-0.2	344.6
14	34.6	121.0	34.9	0.3	349.7
15	34.6	119.7	34.5	-0.1	346.0
16	34.6	116.0	33.5	-1.1	335.5
17	34.6	121.8	35.1	0.5	352.0
18	34.6	120.8	34.7	0.1	348.0
19*	34.7	125.5	36.1	1.4	361.7
20	34.7	117.3	33.9	-0.8	338.0
21	34.8	123.1	35.4	0.6	353.7
22	34.8	121.7	35.1	0.3	349.7
23	34.8	124.8	35.8	1.0	357.8
24*	34.8	124.2	35.7	0.9	356.9
25	34.8	123.2	35.5	0.7	354.0
26	34.9	121.5	35.0	0.1	348.1
27*	35.0	128.0	36.8	1.8	365.7
28	35.0	119.8	34.5	-0.5	342.3
29*	35.1	123.2	35.5	0.4	351.0

Table 6 (Continued)

Sample[1]	Observed crude protein (N x 6.25) a	DBC[2] mg/g meal	Predicted crude protein b	Difference b-a	DBCP[3] mg/g protein
30	35.1	121.7	35.1	0	346.7
31	35.1	125.8	36.2	1.1	358.4
32	35.1	124.1	35.7	0.6	353.6
33	35.2	121.6	35.0	-0.2	345.5
34	35.2	121.7	35.1	-0.1	344.7
35	35.3	120.2	34.7	-0.6	340.5
36	35.3	124.5	35.8	0.5	352.7
37	35.4	123.5	35.5	0.1	348.9
38*	35.4	118.0	34.1	-1.3	333.3
39	35.4	123.0	35.4	0	347.5
40*	35.4	117.7	34.0	-1.4	332.5
41*	35.4	119.3	34.4	-1.0	337.0
42	35.4	128.0	36.8	1.4	361.6
43	35.4	123.7	35.6	0.2	349.4
44	35.4	122.1	35.2	-0.2	344.9
45	35.4	122.4	35.2	-0.2	345.8
46	35.5	123.3	35.5	0	347.3
47	35.5	128.0	37.0	1.5	363.4
48*	35.5	117.1	33.8	-1.7	329.9
49	35.6	120.5	34.7	-0.9	338.5
50	35.6	125.2	36.0	0.4	351.7
51	35.6	122.2	35.2	-0.4	343.3
52	35.6	123.2	35.5	-0.1	346.1
53	35.6	124.0	35.7	0.1	348.3
54	35.7	121.6	35.0	-0.7	340.6
55	35.7	123.3	35.5	-0.2	345.4
56*	35.7	115.6	33.4	-2.3	323.8
57	35.7	127.6	36.7	1.0	357.4
58	35.8	126.0	36.2	0.4	352.0
59	35.8	124.5	35.8	0	347.8
60	35.8	125.0	35.9	0.1	349.2
61	35.8	121.5	35.0	-0.8	339.4
62	35.8	120.5	34.7	-1.1	336.6
63*	35.8	122.4	35.2	-0.6	341.9

Table 6 (Continued)

Sample[1]	Observed crude protein (N x 6.25) a	DBC[2] mg/g meal	Predicted crude protein b	Difference b-a	DBCP[3] mg/g protein
64	35.8	126.5	36.4	0.6	353.4
65	35.8	124.	35.7	-0.1	346.4
66	35.8	123.6	35.6	-0.2	345.3
67	35.9	126.8	36.4	0.5	353.2
68	35.9	123.8	35.6	-0.3	344.8
69	35.9	126.8	36.4	0.5	353.2
70	35.9	124.4	34.8	-0.1	346.5
71	35.9	121.0	34.9	-1.0	337.0
72	36.0	121.5	35.0	-1.0	337.5
73	36.0	120.6	34.8	-1.2	335.0
74	36.1	123.1	35.4	-0.7	341.0
75	36.1	125.7	36.1	0	348.2
76	36.1	127.3	36.6	0.5	352.6
77	36.1	125.0	35.9	-0.2	346.3
78	36.2	127.2	36.5	0.3	351.4
79	36.3	125.3	36.0	-0.3	345.3
80	36.3	123.2	35.5	-0.8	339.4
81	36.3	127.3	36.6	0.3	350.7
82	36.3	128.1	36.8	0.5	352.9
83	36.3	127.3	36.6	0.3	350.7
84	36.4	123.8	35.6	-0.8	340.1
85	36.4	124.9	35.9	-0.5	343.1
86	36.5	124.6	35.8	-0.7	341.4
87	36.5	130.0	37.3	0.8	356.2
88	36.5	127.0	36.5	0	347.9
89	36.5	129.0	37.0	0.5	353.4
90	36.5	128.1	36.8	0.3	351.0
91	36.6	128.1	36.8	0.2	350.0
92	36.6	126.0	36.2	-0.4	344.3
93	36.7	130.6	37.5	0.8	344.9
94	36.7	128.4	36.9	0.2	349.9
95*	36.8	124.0	35.7	-1.1	337.0
96	36.8	121.8	35.1	-1.7	331.0
97	36.8	124.4	35.8	-1.0	338.0

Table 6 (Continued)

Sample[1]	Observed crude protein (N x 6.25) a	DBC[2] mg/g meal	Predicted crude protein b	Difference b-a	DBCP[3] mg/g protein
98*	36.9	130.8	37.5	0.6	354.5
99	36.9	128.0	36.8	-0.1	346.9
100	37.0	128.4	36.9	-0.1	347.0
101	37.0	129.1	37.1	0.1	348.9
102	37.0	125.2	36.0	-1.0	338.4
103*	37.0	130.0	37.3	0.3	351.4
104	37.0	125.4	36.1	-0.9	338.9
105	37.0	129.0	37.0	0	348.6
106	37.1	126.0	36.2	-0.9	339.6
107	37.1	129.6	37.2	0.1	349.2
108	37.3	124.3	35.8	-1.5	333.2
109	37.3	129.7	37.2	0.1	348.0
110	37.3	125.0	35.9	-1.4	335.1
111	37.4	128.7	36.9	-0.5	344.1
112*	37.4	128.4	36.9	-0.5	343.3
113*	37.6	124.1	35.7	-1.9	330.1
114	37.7	128.6	36.9	-0.8	341.1
115	37.7	132.8	38.1	0.4	352.3
116	37.7	130.4	37.4	-0.3	345.9
117*	37.9	130.6	37.5	-0.4	344.6
118	37.9	127.2	36.5	-1.4	335.6
119	37.9	134.1	38.4	0.5	353.8
120*	38.0	127.2	36.5	-1.5	334.7
121	38.1	129.3	37.1	-1.0	339.4
122	38.1	130.8	37.5	-0.6	343.3
123	38.2	134.5	38.5	0.3	352.1
124	38.4	134.5	38.5	0.1	350.3
125*	38.6	135.5	38.8	0.2	351.0
126	39.0	136.1	38.9	-0.1	349.0
Mean	35.93	124.64	35.85	-	347.00

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[1] Samples with asterisk were selected for further basic amino acids and available lysine analyses.

[2] Dye-binding capacity of rapeseed meal.

[3] Dye-binding capacity of protein of rapeseed meal.

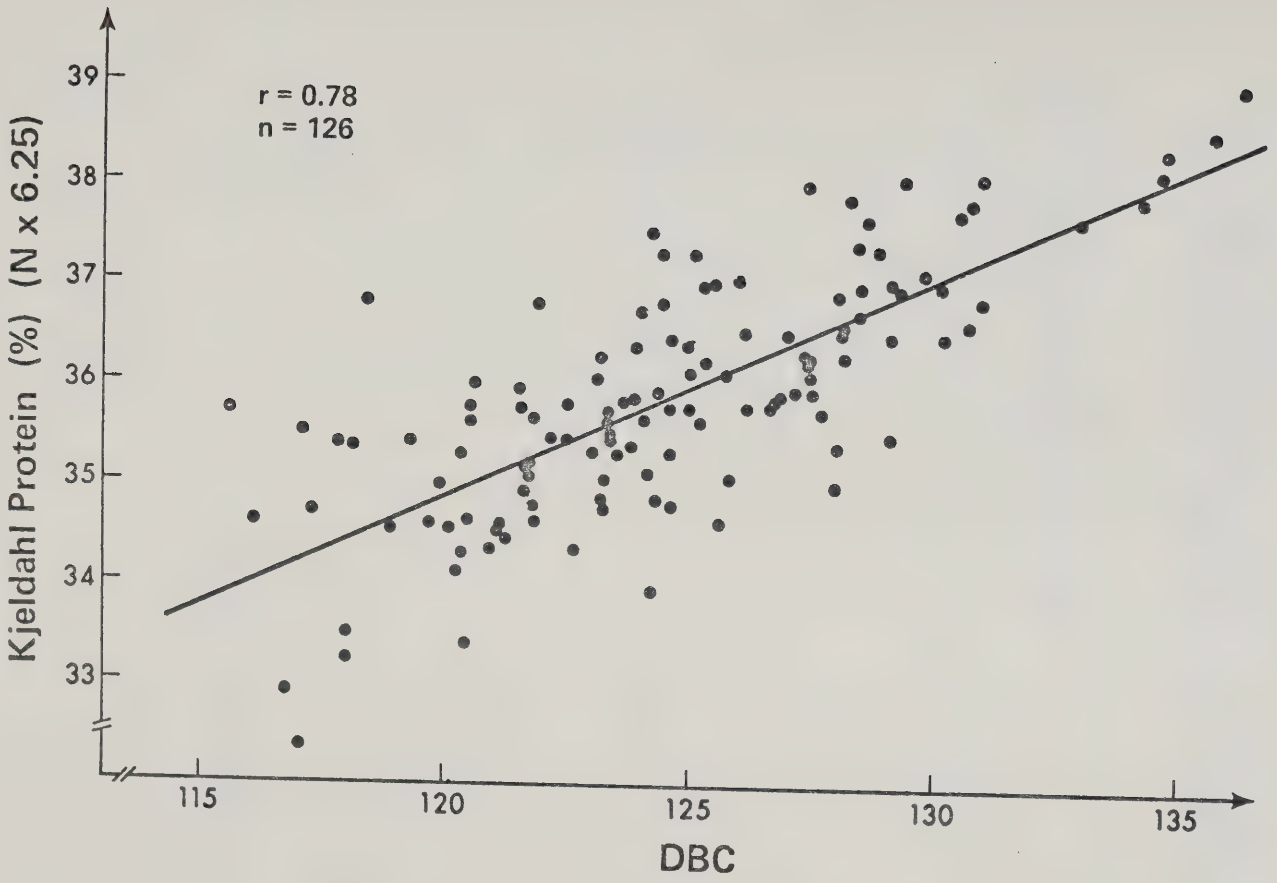

Fig. 4 Relationship between Kjeldahl protein contents and dye-binding capacities (mg Acid Orange 12/g meal) of commercial rapeseed meals.

Table 7

Frequency Distribution of the Differences Between Predicted Protein Percents by the Dye-Binding Capacity Equation and Observed Crude Protein Percents

Difference between predicted and observed values (%)	Frequency
-2.6 to -2.2	1
-2.2 to -1.8	1
-1.8 to -1.4	7
-1.4 to -1.0	11
-1.0 to -0.6	14
-0.6 to -0.2	18
-0.2 to 0.2	30
0.2 to 0.6	27
0.6 to 1.0	9
1.0 to 1.4	5
1.4 to 1.8	3

The reasons(s) for the differences between the predicted protein percents and the analysed values are not known but might be due to the presence of RSMs amongst the samples that had total basic amino acid contents higher or lower than what might be considered as normal. To examine this possibility, the amount of Acid Orange 12 which bound with one gram of RSM protein was calculated for the samples screened. This was done by dividing the DBC of each meal by its corresponding protein percent and the value was designated as the dye-binding capacity of the protein (DBCP) to distinguish it from the DBC of RSM. The data are shown in Table 6. It was found that the DBCP of the RSM samples ranged from 323.8 to 365.7 mg Acid Orange 12 per g protein and average 347 mg per g protein. The frequency distribution of the DBCP values are shown in Table 8.

It would appear from Table 8 that RSM protein usually binds with Acid Orange 12 in the range of 335-355 mg per g protein. However, samples which bound less than 335 or more than 355 mg of Acid Orange 12 per g protein were noted and amounted to approximately 20% of the samples. Since Acid Orange 12 binds only with basic groups of protein, the observation of RSMs with atypical DBCP values suggested that some of the RSM samples contained total basic amino acids in amounts that deviated from usual amounts present in RSM protein. Of the basic amino acids, lysine is known to be most susceptible to destruction by heat treatment. It seemed possible, therefore, that some of the variation noted in the DBCP of the samples

Table 8

Frequency Distribution of the Dye-Binding Capacity of the Protein of Rapeseed Meal

Dye-Binding Capacity of the protein, mg Acid Orange 12/g protein	Frequency
370 to 365	2
365 to 360	5
360 to 355	8
355 to 350	31
350 to 345	35
345 to 340	19
340 to 335	17
335 to 330	7
330 to 325	1
325 to 320	1

of RSM may have reduced the lysine content in the protein of the meals as a result of excessive heat treatment during commercial processing of the rapeseed. As a consequence, it was decided to further examine the relationship between DBCP and the basic amino acid composition of the RSM samples. Twenty-one RSMs covering a wide range of DBCP were selected from the 126 samples for the study. The basic amino acid contents of the meals are presented in Table 9. Correlation coefficients relating the various parameters recorded or calculated are shown in Table 10.

From the correlation coefficients tabulated, the total basic amino acid contents (TBAA) were found to correlate significantly with the DBCP of the RSMs (r = 0.86, Table 10). Of the three basic amino acids analysed, the TBAA was closely related to the lysine and arginine levels but was little affected by the histidine level of the protein. Correlation between DBCP and lysine level of the protein was also highly significant (r = 0.84, Table 10). The lysine level of the selected RSMs decreased from 6.28 to 4.78 g/16g N whereas the DBCP dropped from 365.7 to 323.8 mg Acid Orange 12 per g protein (Table 9).

The results obtained from the basic amino acids analysis support the earlier assumption that the amount of dye bound to one gram of RSM protein was largely determined by the number of basic groups present in the protein available for binding. Consequently the difference between the observed and predicted protein percent varied among the RSM samples

Table 9

Crude Protein, Basic Amino Acids, Dye-Binding Capacities and Available Lysine of Selected Rapeseed Meals

Sample	Observed crude protein (N x 6.25)	DBC mg/g meal	Basic amino acids (g/16 g N)				DBCP mg/g meal	Available lysine	
			Lysine	Arginine	Histidine	TBAA[1]		mg/g meal	mg/g protein
1	32.4	117.1	6.22	5.66	2.74	14.62	361.4	16.3	50.3
2	34.0	124.2	6.06	5.86	2.92	14.82	365.3	16.6	48.9
3	34.7	125.5	6.22	5.84	2.87	14.93	361.7	17.5	50.5
4	35.0	128.0	6.28	6.16	2.77	15.21	365.7	19.2	54.7
5	34.8	124.2	6.02	5.77	3.06	14.85	356.9	18.7	53.7
6	36.9	130.8	6.10	5.79	2.85	14.82	354.5	21.4	57.7
7	33.2	118.0	5.92	5.81	2.68	14.41	355.4	16.1	48.6
8	35.1	123.2	6.02	5.99	2.84	14.85	351.0	17.1	48.8
9	38.6	135.5	6.16	5.87	2.79	14.82	351.0	19.8	52.5
10	37.0	130.0	5.76	5.72	2.89	14.37	351.4	19.3	52.1
11	37.9	130.6	6.24	6.01	2.83	15.08	344.6	18.9	50.0
12	37.4	128.4	5.44	5.69	2.85	13.97	343.3	17.2	45.9
13	35.8	122.4	5.38	5.57	2.91	13.86	341.9	17.5	49.0
14	36.7	124.0	5.36	5.41	2.55	12.43	337.9	16.6	46.1
15	35.4	119.3	5.70	5.72	2.70	14.12	337.0	17.7	50.1
16	38.0	127.2	5.83	5.66	2.66	14.15	334.0	16.6	43.6
17	35.4	118.0	5.10	5.48	2.99	13.57	333.3	14.1	39.6
18	35.4	117.7	5.22	5.36	2.69	13.27	332.5	15.3	43.1
19	37.6	124.1	5.60	5.66	2.66	13.92	330.0	17.0	45.1
20	35.5	117.1	5.24	5.44	2.66	13.34	329.9	13.9	39.1
21	35.7	115.6	4.78	5.17	2.67	12.62	323.8	13.5	37.8

[1] Total basic amino acids.

Table 10

Correlation Coefficients Between Values Reported in Table 9

	Observed crude protein (N x 6.25)	DBC mg/g meal	Basic amino acids				DBCP mg/g protein	Available lysine	
			Lysine	Arginine	Histidine	TBAA		mg/g meal	mg/g protein
Observed crude protein (N x 6.25)	1.00	0.67	-0.12	-0.01	-0.12	-0.1	-0.43	0.35	-0.3
DBC mg/g meal	0.67	1.00	0.57	0.61	0.27	0.60	0.38	0.83	0.63
Lysine	-0.12	0.57	1.00	0.89	0.27	0.97	0.84	0.72	0.82
Arginine	-0.01	0.61	0.89	1.00	0.36	0.95	0.76	0.70	0.75
Histidine	-0.12	0.27	0.36	0.36	1.00	0.47	0.46	0.33	0.38
TBAA	-0.10	0.60	0.97	0.95	0.48	1.00	0.86	0.74	0.83
DBCP mg/g protein	-0.43	0.38	0.84	0.76	0.46	0.86	1.00	0.58	0.79
Available lysine									
mg/g meal	0.35	0.83	0.72	0.70	0.33	0.74	0.58	1.00	0.92
mg/g protein	-0.03	0.63	0.82	0.75	0.38	0.83	0.79	0.92	1.00

studied, depending on the amount of dyes bound to a unit weight of protein. Above the average of 347 mg/g protein, the predicted value over-estimated the protein content of the meal and vice versa. The high correlations between the DBCP and the total basic amino acids particularly lysine further suggests that DBCP of a sample is a good measure of the protein quality of the meal.

To verify the protein quality predicted by DBCP, the same RSM samples were assayed for available lysine contents. The available lysine contents of the RSMs, expressed as mg per g meal and mg per g protein, are shown in Table 9. The values have been adjusted for loss of dinitrophenyl-lysine (DNP-lysine) during the acid hydrolysis in the determination. The correlation coefficient between DBC and mg available lysine per g meal of the samples was 0.83 and that between DBCP and mg per g protein was 0.79. In both cases, the correlation coefficients were highly significant ($P<0.01$). The results thus showed agreement in protein quality estimation of the RSM samples by the DBCP and the available lysine methods.

Although numerous regression equations relating a single dye-binding characteristic and Kjeldahl protein percent have been reported for various protein-rich food and feedstuffs, as reviewed previously, few have been critically tested for practical applicability. However, results from numerous comparative studies of the Udy Protein Analyzer and Kjeldahl method reported in the literature were in favour of the use

of the dye-binding method for routine analysis (Olson and Heiges, 1962; Hymowitz et al. 1969; Parial et al. 1970). The use of the DBC determination for estimating the crude protein of RSM appears to over-estimate samples with protein contents below 35% but under-estimated, in most instances, when the protein contents of the samples were above 35%.

The high correlation between DBC and available lysine content of the RSMs was comparable to that reported for fishmeals by Carpenter and Opstvedt (1976). These workers found a high correlation between the DBC and available lysine of eight fishmeals and the protein qualities of these samples were confirmed in chick bioassay. In the present study, the size of the RSM samples received was inadequate to permit their use in biological assays and as a result, the predicted nutritional values of the selected RSMs could not be verified biologically.

4 Summary

The linear equation relating the DBC and Kjeldahl protein of RSM obtained in Experiment 1 was applied to 126 samples of RSM for estimating the protein contents of the meals. Results obtained indicated that the predicted protein percent by the dye-binding technique over-estimated the protein contents of meals in the lower protein range but under-estimated those in the higher protein range. A further study of selected meals revealed, however, that the deviation was caused mainly by the presence of samples having atypical basic amino acid compositions. The dye-

binding capacity of RSM protein was found to correlate highly with the lysine and available lysine contents of the meals, indicating the potential of the use of dye-binding capacity per g protein as a protein quality index for RSM.

c Experiment 3: The Effect of Moist Heat on the Dye-Binding Capacity of Rapeseed Meal with Acid Orange 12

1 Objective

In Experiment 2, it was found that the predicted protein content obtained by using the dye-binding equation underestimated the crude protein percent for some RSM samples. It was felt that this was due to a lower than average level of total basic amino acids in these samples. This was reflected in a reduction in the amount of Acid Orange 12 bound to a unit weight of RSM protein (DBCP). Since excess heat treatment seemed to be the most likely cause of the reduction in basic amino acids in these meals it was decided to study in this experiment the effect of heating meal in an autoclave for various periods of time on the DBC of the resulting meals. The adverse effect on the nutritional quality of the meals was also assessed by chemical determination of the available lysine in the meals.

2 Materials and Methods

Eight commercial RSMs comprised of two Bronowski, two Span and four Tower meals were used in this study. Each meal was divided into seven portions of approximately 1 lb each. Portions of each meal were autoclaved at 121°C for 0, 15, 30, 45, 60, 90 and 120 minutes. Untreated and autoclaved meals were analysed for crude protein and for their DBC with Acid Orange 12 as described previously. RSM samples autoclaved for 0, 30, 60, 90 and 120 minutes were further analysed for available lysine by Booth's (1971) procedure.

3 Results and Discussion

The crude protein contents (N x 6.25) of the samples as determined by the Kjeldahl procedure are presented in Table 11. A small decrease in percentage of protein was noted in a few of the samples autoclaved for 120 minutes.

The uptakes of Acid Orange 12 by the RSMs are tabulated in Table 12 and shown graphically in Fig. 5, plotted against the duration of autoclaving at 121°C. Autoclaving at 121°C for as long as 30 minutes did not affect the ability of the meals to bind Acid Orange 12. Decrease in DBC, however, became apparent when the duration on heat treatment reached 45 minutes and the DBC continued to decline as the treatment was prolonged. The data were analysed by one-way analysis of variance and significant differences among treatment means were compared using Duncan's Multiple Range test (Steel and Torrie, 1960). It was found that the mean DBC of the RSMs autoclaved at 121°C for 90 minutes was significantly lower than those of meals treated for 45 minutes or less ($P<0.05$). Increasing the duration of treatment beyond 90 minutes did not further reduce the DBC of the meals significantly. To determine whether the rates of decrease with time in DBC differed among the eight RSMs studied, statistical analysis of homogeneity of regression coefficients of DBC with time was computed. The results indicated that the Span RSM (S2) showed the largest decline in DBC after 120 minutes of heat

Table 11

Crude Protein Percent of Rapeseed Meals Autoclaved at 121°C for Various Periods of Time

Sample	Autoclaving time, minutes						
	0	15	30	45	60	90	120
Bronowski (B1)	39.5	40.0	39.5	39.6	40.0	40.9	38.8
Bronowski (B2)	37.5	36.6	37.7	37.0	37.1	37.4	36.3
Span (S1)	35.6	36.0	36.0	36.1	36.1	36.3	36.2
Span (B2)	35.0	35.1	35.1	34.8	34.8	35.4	34.0
Tower (T1)	36.4	36.6	36.4	36.5	36.2	36.7	35.5
Tower (T2)	36.0	35.7	36.3	35.5	35.4	36.3	35.5
Tower (T3)	34.6	35.3	35.5	34.5	34.8	35.4	35.8
Tower (T4)	30.1	29.0	29.8	29.8	29.4	30.6	30.0

Table 12

Dye-Binding Capacity[1] of Rapeseed Meals Autoclaved at 121°C for Various Period of Time

Sample	Autoclaving time, minutes						
	0	15	30	45	60	90	120
Bronowski (B1)	140.1	139.5	139.6	137.4	135.3	127.8	129.0
Bronowski (B2)	131.5	132.8	131.5	126.8	124.0	117.0	112.6
Span (S1)	125.5	124.0	121.6	118.6	115.5	108.1	102.8
Span (S2)	124.5	123.8	122.7	120.3	115.9	106.1	97.3
Tower (T1)	124.5	127.0	120.6	121.2	115.2	109.1	102.6
Tower (T2)	121.6	123.4	121.2	117.5	112.9	106.9	103.7
Tower (T3)	124.2	123.8	119.9	117.5	115.3	100.4	101.9
Tower (T4)	103.7	103.1	103.8	100.7	96.1	88.7	85.7
Mean[2]	124.4^{a}	124.7^{a}	122.6^{a}	120.0^{a}	116.3^{ab}	108.0^{bc}	104.4^{c}

[1] Expressed as mg Acid Orange 12 bound per gram of RSM.

[2] Means with the same superscript were not significantly different ($P<0.05$).

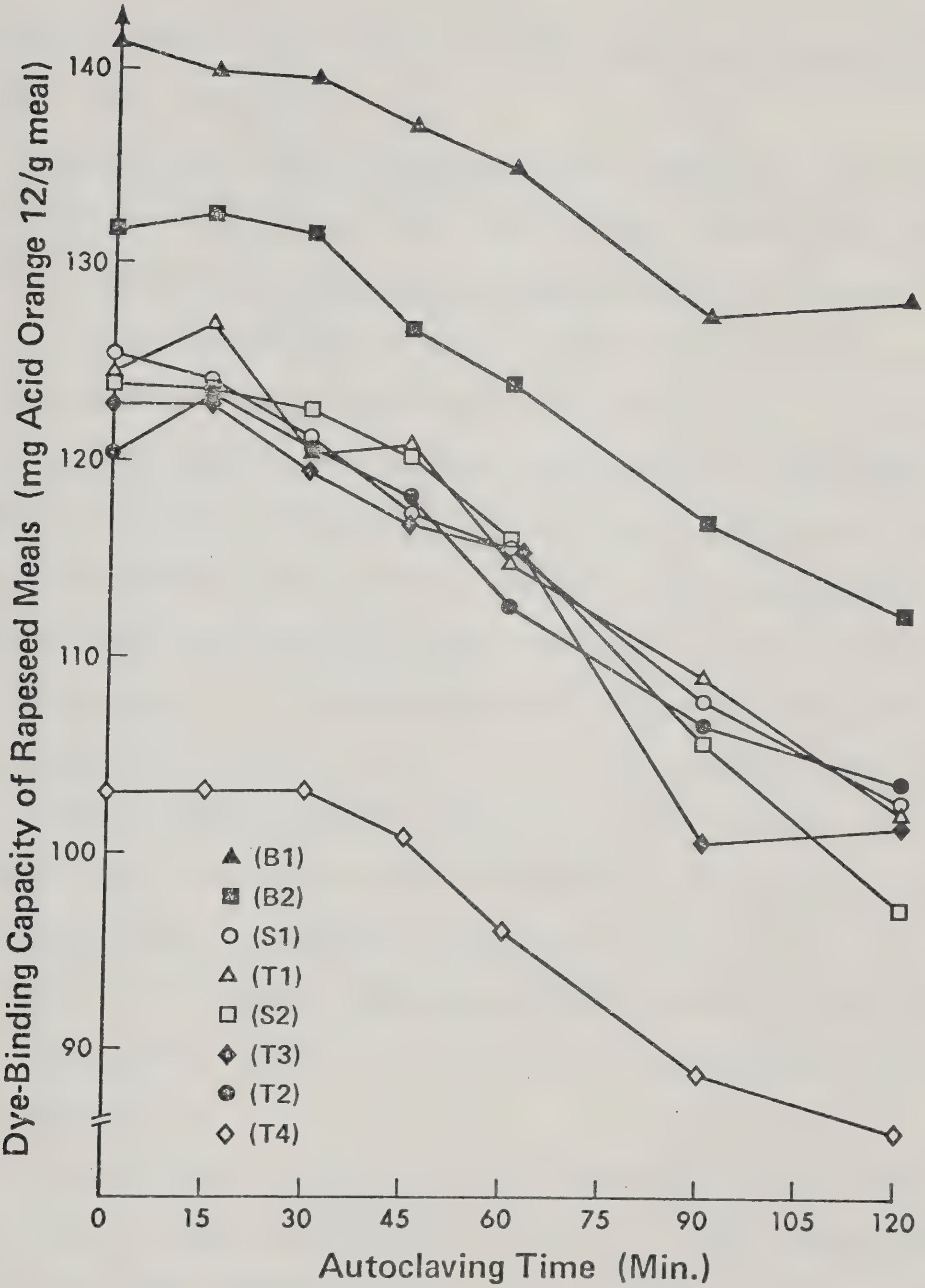

Fig. 5 Relationship between the dye-binding capacity of rapeseed meal and the duration of autoclaving of the meal at 121°C.

treatment whereas the Bronowski RSM (B1), was least affected by the heat treatment.

The protein quality status of the RSMs was also studied. The DBCP calculated for the treated RSMs (Table 13) paralleled that of the DBC of the meals. The mean DBCP decreased from 350 mg dye/g protein for the untreated RSMs to 295 mg dye/g protein for meals autoclaved for 120 minutes. As was the case for DBC, decreases in DBCP were noted in some RSMs treated for only 30 minutes. Statistical analysis of the DBCP data showed that the mean DBCP of RSMs autoclaved for 45 minutes was significantly lower than those of meals treated for 15 minutes or receiving no treatment but higher than those autoclaved for 60, 90 and 120 minutes ($P<0.05$).

These results clearly indicate that prolonged heat treatment of RSM reduces both the DBC and the DBCP of meals without materially reducing the crude protein (N x 6.25) contents of the meals. Hence the DBC or DBCP of such RSMs would not yield a reliable estimate of the protein contents of the RSMs.

In the study on the effect of autoclaving the RSMs for 0, 30, 60, 90 and 120 minutes at 121°C, it was found (Tables 14 and 15) that the available lysine contents of the RSMs were significantly reduced when treated for 60 minutes and continued to decline at 90 and 120 minutes.

Correlation coefficient between DBC and available lysine (Tables 12 and 14) of the RSMs was 0.84 and that between DBCP and mg available lysine/g protein (Tables 13 and 15) was 0.94.

Table 13

Dye-Binding Capacity[1] of the Protein of Rapeseed Meals Autoclaved at 121°C for Various Periods of Time

Sample	Autoclaving time, minutes						
	0	15	30	45	60	90	120
Bronowski (B1)	354.7	349.1	353.5	346.8	338.2	312.6	332.5
Bronowski (B2)	350.6	362.6	348.5	342.6	334.0	312.8	309.8
Span (S1)	352.5	344.3	337.8	328.2	320.1	297.4	283.6
Span (S2)	355.7	352.7	349.5	345.2	333.3	299.9	286.2
Tower (T1)	342.0	346.8	331.5	331.9	318.4	297.2	288.7
Tower (T2)	337.8	345.7	334.1	331.6	319.0	294.5	292.1
Tower (T3)	358.9	350.1	337.9	341.0	331.7	283.9	284.5
Tower (T4)	344.5	355.2	348.1	337.7	327.0	289.6	285.9
Mean[2]	349.6^a	350.9^a	342.6^{ab}	338.1^b	327.7^c	298.5^d	295.4^d

[1] Expressed as mg Acid Orange 12 bound per g of RSM protein.

[2] Means with the same superscript were not significantly different ($P<0.05$).

Table 14

Available Lysine Content[1,2] of Rapeseed Meals Autoclaved at 121°C for Various Periods of Time

	Autoclaving time, minutes				
Sample	0	30	60	90	120
Bronowski (B1)	22.6	20.2	17.4	14.0	12.7
Bronowski (B2)	20.2	16.9	14.8	11.9	11.3
Span (S1)	18.2	14.2	12.0	7.6	6.0
Span (S2)	18.1	17.3	12.5	8.3	5.4
Tower (T1)	17.6	16.2	12.7	10.8	8.6
Tower (T2)	16.8	16.4	15.4	11.7	8.9
Tower (T3)	19.4	17.0	12.5	7.3	6.5
Tower (T4)	15.7	13.2	10.6	9.3	7.4
Mean[3]	18.6^a	16.4^a	13.5^b	10.1^c	8.3^c

[1] Expressed as mg available lysine per g of RSM.

[2] The correlation coefficient between the tabulated values and the corresponding dye-binding capacities of the meals was 0.84.

[3] Means with the same superscript were not signficantly different ($P<0.05$).

Table 15

Available Lysine Contents[1,2] of the Protein of Rapeseed Meals Autoclaved at 121°C for Various Periods of Time

Sample	Autoclaved time, minutes				
	0	30	60	90	120
Bronowski (B1)	57.1	51.1	43.4	34.2	32.8
Bronowski (B2)	53.9	44.9	39.7	31.7	31.0
Span (S1)	51.2	39.3	33.3	21.0	16.0
Span (S2)	51.8	49.3	36.0	23.6	15.9
Tower (T1)	48.3	44.4	35.1	29.5	24.1
Tower (T2)	46.7	45.3	43.5	32.1	25.0
Tower (T3)	56.0	48.0	35.9	32.1	25.0
Tower (T4)	52.0	44.3	36.1	30.4	24.5

[1] Expressed as mg available lysine per g of RSM protein.

[2] The correlation coefficient between the tabulated values and the corresponding dye-binding capacities of protein of the meals was 0.94.

Both coefficients were highly significant ($P<0.01$).

Although the highly positive correlation between DBC and available lysine seemed to suggest that the ability of the RSM to bind Acid Orange 12 was a reliable means of detecting the decrease in protein quality of the RSMs with increasing time of autoclaving, the degree of protein damage measured by the dye-binding method and by the available lysine determination for the same meal seemed to differ. In comparing the decreases in DBC and available lysine of the autoclaved RSMs, the DBC and mg available lysine/g meal were expressed as percentages of those of the corresponding unheated meals and the results are presented in Table 16. From the tabulated percentages, it will be seen that the available lysine levels in the RSMs declined more rapidly than the DBCs for the same meals. The available lysine contents of the meals were decreased appreciably by autoclaving the meals for as short a time as 30 minutes while the DBCs of the meals were only slightly affected. The differences became more marked as the duration of heat treatment increased. The mean reduction from 120 minutes of autoclaving was 16% in DBC as compared to 55% in available lysine content. This difference might be caused by the continued binding between Acid Orange 12 and the damaged basic amino groups through some unknown secondary mechanisms other than electrovalent attraction.

Several reports in the literature have demonstrated the adverse effect of heat treatment either by autoclaving or by oven-heating on the dye-binding ability of various feedstuffs

Table 16

Comparison of Dye-Binding Capacities and Available Lysine Contents of Rapeseed Meals Expressed as Percentages of the Untreated Meals[1]

Sample	Autoclaving time, minutes				
	0	30	60	90	120
Bronowski (B1)	100	99.6 (89.5)	96.6 (77.0)	91.2 (62.0)	92.0 (56.4)
Bronowski (B2)	100	100.0 (84.0)	94.3 (73.1)	89.0 (58.8)	85.6 (55.9)
Span (S1)	100	96.9 (77.7)	92.0 (66.0)	86.1 (41.9)	81.9 (31.7)
Span (S2)	100	98.5 (95.5)	93.1 (69.0)	85.2 (46.0)	78.2 (29.7)
Tower (T1)	100	96.9 (91.8)	92.5 (72.1)	87.7 (61.6)	82.4 (48.7)
Tower (T2)	100	99.6 (97.6)	92.8 (91.4)	87.9 (69.3)	85.2 (52.8)
Tower (T3)	100	96.5 (87.9)	92.9 (64.4)	80.9 (69.3)	82.1 (33.7)
Tower (T4)	100	100.1 (84.4)	92.7 (67.7)	85.6 (59.4)	82.7 (46.9)
Mean	100	98.5 (88.6)	93.4 (72.6)	86.7 (54.6)	83.7 (44.5)

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[1] Percents in parenthesis refer to available lysine expressed as percentage of the values for the untreated meals.

(Choppe and Kratzer, 1963; Moran et al., 1963; Udy, 1971). The adverse effect of autoclaving on the DBC of RSMs recorded in the present study agreed well with the findings of the above workers. As an example, Udy (1971) reported decrease in DBCP of soybean meal protein from 378 to 367 mg Acid Orange 12/g protein after 1 hour of oven heating at 130°C. This is equivalent to a reduction of 3%. For RSMs autoclaved for 1 hour at 121°C, the mean DBCP decreased from 349.6 to 327.7 mg Acid Orange 12/g protein, a reduction of 6.3%. The close correlation between the amount of Acid Orange 12 bound by the protein of commercial RSM and the available lysine of the protein of the same RSMs also compared favourably with findings for other feedstuffs (Hurrell and Carpenter, 1975; Carpenter and Opstvedt, 1976). The differences in rates of decrease in DBC and available lysine as a result of autoclaving of RSM agree with the findings reported by Hurrell and Carpenter (1975). They found that groundnut meals heated in an autoclave at 121°C for 1 and 4 hours bound 1030 and 920 mmole Acid Orange 12/kg crude protein as compared to 1060 mmole for the unheated meal. The decreases were equivalent to 3 and 13% of the control value. For available lysine the corresponding decreases were 8 and 50% respectively. The basic amino acids of the RSMs were not determined in the present study and the reduction of number of basic groups available for binding with Acid Orange 12 as a result of autoclave heating was not known.

4 Summary

The dye-binding capacities (DBC) and protein quality of RSMs which were heated in an autoclave at 121°C for various periods of time were studied. The results indicated that heating RSMs adversely affected their ability to bind Acid Orange 12. In this regard, a decrease in the DBC of the meals was noted with as short an autoclaving period as 30 minutes and the DBC of the meals decreased as the autoclaving time increased.

The protein quality of the RSMs, as estimated by the DBC of the protein of the meals also decreased with increasing time of autoclaving. In this connection the DBCP of the RSMs was significantly decreased after 45 minutes of heating in the autoclave and continued to decline as the duration of heat treatment was prolonged.

The protein quality of the RSMs, as measured by available lysine contents of the meals, decreased with increasing length of time that the RSMs were heat treated. The percentage decrease in available lysine as a result of the heat treatments was greater than the percentage decrease in DBCP. This would suggest that where protein quality predictions are made on heat-damaged feedstuffs, measurement of available lysine would give a more reliable measure of protein quality than measurement of the DBCP.

d Experiment 4: Evaluation of Protein Quality of Commercially Produced and Laboratory Heat-Damaged Rapeseed Meals by the Dye-Binding Method and by Biological Assay with Chicks

1 Objectives

The purpose of this study was to examine the DBCPs of both commercially produced RSMs and meals which were deliberately heat-damaged in the laboratory and to determine whether a relationship existed between the protein quality index as predicted by the dye-binding method and by biological assay using chicks as the experimental animals.

2 Materials and Methods

i Protein quality evaluation of rapeseed meals by the dye-binding method

Eighty commercial RSMs obtained from four processing plants (20 from each) were screened for their DBCs with Acid Orange 12. The meals were collected over a 10 day period with one sample obtained in the morning and one in the evening of each day. One sample from each processor on each day was provided in quantity adequate for biological assay purpose. The latter were samples of evening collections from three plants and morning collections from the remaining plant.

In addition small quantities of Tower RSM were deliberately heat-damaged by autoclaving at 121°C for 1/2, 1, 2 and 4 hours. The autoclaved meals, as well as the original sample of Tower RSM, were tested for DBC with Acid Orange 12.

The crude protein contents of all RSM samples were determined by the Kjeldahl procedure (A.O.A.C., 1970).

ii Biological evaluation of the protein quality of rapeseed meals by the total protein efficiency method

Two chick growth trials were conducted to study the relationship between the protein quality of the RSMs as predicted by dye-binding and by chick growth. The chick growth trial employed for evaluating the protein quality was the total protein efficiency (TPE) method developed by Woodham (1967).

In growth Trial 1, the selection of the commercial RSMs for the biological assay was restricted by several factors and as a consequence was difficult to make. Firstly although 80 RSMs were screened in the DBC study only 40 of them were available in sufficient quantity for biological evaluation. Secondly, the differences found in the DBCPs of RSMs collected at one plant in the 10 day collection period were relatively small. For the ten samples received from this processor (Plant 1, Table 21) the difference was only approximately 10 mg dye per g protein. The differences for the remaining three processors were about 20 mg dye per g protein. The commercial RSMs eventually selected for the chick growth trial were the eight samples representing the pair of meals with the highest and lowest DBCP values from each processing plant. In the formulation of the experimental rations, the RSMs which showed the highest DBCP from Plants 2 and 3 were, however, found inadequate to provide the 12% protein required in the

test ration. In these cases, the meals having the second highest DBCP values were used.

In growth Trial 2, the five Tower RSMs were used in the bioassay.

The experimental rations used in the two trials were formulated to contain 18% crude protein of which 12% was provided by the test supplement (RSM) and the remaining 6% by the cereal grains, corn, wheat and wheat shorts (Tables 17 and 18). The rations were isonitrogenous and approximately isocaloric.

In Trial 1, 240 two week old chicks were selected from a flock of Hubbard broiler-type male chicks (Dominant White male x White Plymouth Rock female) which had been fed a balanced starter ration. The selected chicks were allotted into groups of 10 birds each such that the total body weight of birds in each group was approximately the same. Three groups of chicks were fed each of the eight rations shown in Table 17.

In Trial 2, 150 Shaver broiler-type male chicks were selected as described above and three groups of chicks were fed each of the five rations shown in Table 18.

At the end of the 14 day experimental periods, the feed intakes and body weights of the groups were recorded. The total protein efficiency (TPE) of the test feedstuffs was calculated as the weight gain of the chicks divided by the total weight of the protein eaten by the chicks.

Table 17

Composition of Rations Used in Growth Trial 1

Ingredients	Rations 1	2	3	4	5	6	7	8
Basal[1]	63.00	63.00	63.00	63.00	63.00	63.00	63.00	63.00
RSM[2]	34.03	34.33	36.50	34.55	33.59	31.55	36.70	34.55
Cellulose	2.97	2.67	0.50	2.45	3.41	5.45	0.30	3.15
Calculated Analysis:								
ME, kcal/kg	2592	2597	2611	2631	2584	2575	2631	2601
Crude protein, %	18.01	18.01	18.01	18.01	18.01	18.01	18.01	18.01
Ca, %	1.04	1.05	1.05	1.06	1.04	1.04	1.06	1.05
P, %	0.79	0.79	0.80	0.81	0.78	0.78	0.81	0.79

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[1] Contained per 63 kg; ground corn, 45; ground wheat, 9; wheat shorts, 4.37; stabilized fat, 1; ground limestone, 1.5; dicalcium phosphate, 1.25; iodized salt, 0.35; manganous oxide, 0.02; zinc oxide, 0.01; and vitamin premix, 0.5 kg. Vitamin premix supplied per kg ration: vitamin A, 5000 IU; vitamin D_3, 1000 IU; Vitamin E, 10 IU; vitamin K, 1 mg; vitamin B_{12}, 0.01 mg; riboflavin, 5 mg; pyridoxine hydrochloride, 6.2 mg; folic acid, 0.55 mg; calcium pantothenate, 15.4 mg; nicotinic acid, 44 mg; biotin, 0.1 mg.

[2] RSM in rations 1 and 2 were from Plant 1, 3 and 4 from Plant 2, 5 and 6 from Plant 3 and 7 and 8 from Plant 4.

Table 18

Composition of Rations Used in Growth Trial 2

	Rations[1]				
Ingredients	1	2	3	4	5
Basal[2]	63.00	63.00	63.00	63.00	63.00
RSM[3]- 0 hr[4]	30.73	-	-	-	-
RSM - 1/2 hr	-	30.73	-	-	-
RSM - 1 hr	-	-	30.73	-	-
RSM - 2 hr	-	-	-	30.73	-
RSM - 4 hr	-	-	-	-	30.73
Cellulose	6.27	6.27	6.27	6.27	6.27

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[1] Calculated analysis: ME, 2625 kcal/kg; crude protein, 18.5; Ca, 1.02%; P, 0.79%.

[2] See Table 17.

[3] The rapeseed meal used was Tower meal which contained 39.04% of crude protein as determined by Kjeldahl method.

[4] Time autoclaved at 121°C.

The basic amino acids of the RSM samples selected for the protein quality evaluation in both trials and the amino acids composition of the rations used in the two trials were analysed by the procedure described in Experiment 1.

3 Results and Discussion

i Protein quality evaluation of rapeseed meals by the dye-binding method

The crude protein contents estimated by the Kjeldahl procedure and the DBCs of the 80 commercial RSMs are summarized in Tables 19 and 20. The data were subjected to a simple correlation analysis and it was found that the correlation coefficient between the DBCs and crude protein contents of the RSMs was highly significant ($r = 0.84$).

To evaluate the protein quality of the RSMs, the DBCPs were calculated and tabulated in Table 21. The data on DBCP were analysed statistically by multiway analysis of variance (Steel and Torrie, 1960). From the results shown in Table 22, it is apparent that there were no significant differences in the amounts of Acid Orange 12 bound to the protein of RSMs produced by the different processing plants. However, the DBCPs of the RSMs collected in the morning and the evening were significantly different ($P<0.05$). The DBCPs of the RSMs collected from the same plant over the 10 day period were not tested for their difference among day of collection since only single samples were obtained in the morning or evening within the same day.

Table 19

Crude Protein Contents[1] of Commercial Rapeseed Meals from Four Processing Plants

	Plant							
	1		2		3		4	
Day	Morning	Evening	Morning	Evening	Morning	Evening	Morning	Evening
1	33.8	35.2	35.2	35.7	35.8	35.5	33.7	34.1
2	35.1	35.3	35.4	34.9	36.3	35.7	34.4	34.2
3	34.7	33.8	32.8	34.7	33.5	35.5	33.7	31.6
4	33.1	34.9	31.3	33.7	34.3	38.0	33.6	34.0
5	34.7	34.7	32.0	34.8	36.8	36.3	33.4	32.8
6	34.0	34.8	34.5	33.9	36.4	36.3	32.7	33.5
7	35.3	34.0	34.7	35.8	36.6	35.9	33.5	33.1
8	35.9	35.7	31.5	32.9	36.2	34.7	33.5	34.0
9	35.5	36.1	33.3	34.3	35.5	36.6	33.9	33.1
10	35.8	35.7	34.2	34.9	37.7	36.3	33.9	33.6

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[1] Expressed in %.

Table 20

The Dye-Binding Capacities[1,2] of Commercial Rapeseed Meals with Acid Orange 12

	Plant							
	1		2		3		4	
Day	Morning	Evening	Morning	Evening	Morning	Evening	Morning	Evening
1	121.3	124.6	122.2	122.8	127.8	123.7	118.5	117.2
2	125.6	125.9	123.6	117.8	127.8	126.7	117.8	117.5
3	123.1	117.4	116.2	117.1	126.4	123.4	116.5	111.5
4	117.8	123.2	110.8	121.9	129.5	127.5	116.9	116.2
5	124.4	124.2	116.8	124.0	129.7	126.4	117.3	115.1
6	120.8	120.9	124.7	122.0	128.9	127.0	117.8	116.8
7	124.3	121.8	125.7	123.0	127.5	125.7	116.3	117.8
8	127.2	126.2	115.8	114.3	125.8	123.2	117.4	120.6
9	125.6	125.2	118.8	120.8	126.8	125.3	118.4	115.9
10	126.4	125.3	119.9	123.2	123.4	123.2	118.6	116.7

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[1] Expresses as mg Acid Orange 12 bound per gram meal.

[2] The correlation coefficient between the DBCs and the crude protein contents of the meals was 0.84 and was highly significant ($P<0.01$).

Table 21

The Dye-Binding Capacities[1] of the Protein of Commercial Rapeseed Meals with Acid Orange 12

	Plant							
	1		2		3		4	
Day	Morning	Evening	Morning	Evening	Morning	Evening	Morning	Evening
1	358.6	353.9	347.3	344.1	357.0	348.8	351.5	343.7
2	358.1	357.0*[2]	349.2	358.3*	352.1	354.8*	342.3*	343.9
3	354.5	347.1*	354.4	337.2*	376.9	347.9	345.1	353.2
4	355.9	352.5	354.5	361.4	377.6	335.3*	348.1	341.6
5	358.3	357.0	364.8	355.9	352.9	348.6	350.9	351.3
6	355.4	347.1	361.9	357.6	353.7	349.7	360.2*	348.9
7	352.2	354.9	362.6	343.3	348.3	350.2	346.8	356.0
8	354.1	353.0	366.4	346.9	347.7	355.4	350.8	355.2
9	353.2	346.4	356.5	351.9	357.0	341.8	349.2	349.9
10	352.3	351.2	350.4	35.38	327.4	339.8	350.4	347.8

[1] Expressed as mg Acid Orange 12 bound per gram protein in the meal.

[2] Rapeseed meals marked with an asterisk were selected for feeding trial 1 with chicks.

Table 22

Analysis of Variance of DBCP of Commercial Rapeseed Meals

Source of variance	Degree of freedom	Sum of square	Mean square	F-value
Plant	3	284.7	94.9	1.91
Day/plant	36	1973.6	49.8	-
Time	1	370.4	370.4	6.55*[1]
Plant x time	3	155.6	51.9	0.92
Time/day/plant	36	2035.7	56.9	-

[1] Significantly different at $P < 0.05$.

The crude protein contents, DBC and DBCP data on the Tower RSMs autoclaved for different periods of time at 121°C are presented in Table 23. Statistically, a highly negative correlation was found between the DBCs of the meals and the duration of autoclave treatment ($r = -0.95$). This result clearly demonstrated the inverse relationship between the length of time the meal was autoclaved and its ability to bind Acid Orange 12. The DBC and DBCP data of the autoclaved meals were not analysed statistically since a single sample of each meal was used in the determination of DBC. However, the amount of Acid Orange 12 bound to the Tower meal autoclaved for only 30 minutes was considerably reduced as compared to that of the untreated Tower meal (136.6 vs 127.8 mg dye/g meal and 349.9 vs 326.9 mg dye/g protein). By the time the meal was heated for 4 hours, the DBC and DBCP values were only 77% of the control value. Thus, the protein quality of these autoclaved meals appeared severely damaged.

ii Biological evaluation of the protein quality of rapeseed meals by the total protein efficiency method

The growth data (Trial 1) are summarized in Table 24. The means for TPE for the eight RSMs evaluated ranged from 2.22 to 2.47. The TPE means were analysed statistically by one-way analysis of variance and it was found that there were no significant differences among the mean TPE values of the eight RSMs evaluated ($P<0.05$), nor was there a significant correlation between the DBCP and the TPE obtained. Since the results of the growth trial failed to show any positive relationship between the protein quality index as predicted

Table 23

Protein Contents and Dye-Binding Capacities of Tower Rapeseed Meals Autoclaved for Different Periods of Time at 121°C

Autoclave treatment hr at 121°C	Crude protein %	DBC, mg dye per g meal	DBCP, mg dye per g protein
0	39.0	136.6[1]	349.9
1/2	39.1	127.8	326.9
1	39.0	119.8	307.3
2	39.0	113.6	291.6
4	39.0	104.0	267.1

[1] Correlation coefficient between DBC and autoclaving time was -0.95 and was highly significant ($P<0.01$).

Table 24

Summary of Results of Growth Trial 1[1]

Ration[2]	DBCP, mg dye/g protein	Weight gain, g/group	Feed intake, g/group	Crude protein eaten,[3] g/group	TPE[4]
1	357.0	3690	8302	1494	2.47
2	347.1	3291	7993	1439	2.29
3	358.3	3322	8198	1476	2.25
4	337.2	3345	8208	1477	2.26
5	354.8	3168	7933	1428	2.22
6	335.3	3647	8467	1524	2.39
7	360.2	3516	8286	1492	2.36
8	342.3	3300	7985	1437	2.30
S.E.M.[5]		±100	±157.3	±28	±0.05
D.F.[6]		16	16	16	16

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[1] The means of weight gain, feed intake, crude protein eaten and TPE were averages of three replicate groups.

[2] See Table 17 for composition of rations.

[3] The amount of crude protein eaten was calculated on the basis of 18% in the ration.

[4] The means were not significantly different ($P<0.05$).

[5] Standard error of the mean.

[6] Degree of freedom.

by the dye-binding method (DBCP) and the TPE measured biologically, the RSMs and rations used were further analysed for their contents of basic amino acids. The analytical results are shown in Table 25. The levels of lysine in the RSMs seemed to be in good agreement with the DBCPs calculated. Within each pair of RSMs from the same plant, the meal having a higher DBCP contained a higher level of lysine expressed as g/16g N. However, neither the levels of lysine in the RSMs nor in the rations explained the lack of relationship between the DBCPs and TPEs. The probable cause could be that despite the differences in DBCPs noted among the meals used in the protein evaluation, the lysine levels (ranged from 5.13 to 6.03 g/16g N) were adequate to support comparable growth.

The data for growth Trial 2, in which the effect of laboratory autoclave treatment on the protein quality of Tower RSM was evaluated, are presented in Table 26. The DBCPs of the meals and the mean TPEs, analysed by a simple correlation analysis, were highly correlated ($r = -0.96$), indicating a decreasing TPE values with decreasing DBCP values. Weight gain and feed intake also declined with declining TPE values. The TPE data were further subjected to one-way analysis of variance followed by Duncan's Multiple Range test (Steel and Torrie, 1960) and it was found that all of the TPE means for the five Tower RSMs were significantly different from each other ($P<0.05$). However, the decrease in protein quality of the RSMs as demonstrated biologically occurred at a faster rate than the decline in DBCP as a function of autoclaving duration.

Table 25

Basic Amino Acid Composition of Rapeseed Meals and of Experimental Rations Used in Growth Trials 1 and 2

Trial[2]	Ration	DBCP of RSM	Basic amino acids in RSM[1]				Basic amino acids in ration[1]		
			Lysine	Histidine	Arginine	TBAA	Lysine	Histidine	Arginine
1	1	357.0	5.90	2.74	5.65	14.29	5.02	2.79	5.36
1	2	347.1	5.81	2.72	5.77	14.30	4.83	2.65	5.25
1	3	358.3	6.03	2.71	5.56	14.30	4.99	2.76	5.17
1	4	337.2	5.70	2.54	5.42	13.62	4.88	2.77	5.23
1	5	354.8	5.43	2.76	5.74	13.93	4.54	2.75	5.05
1	6	335.3	5.21	2.58	5.49	13.28	4.57	2.78	5.22
1	7	360.2	5.39	2.69	5.65	13.73	4.75	2.79	5.22
1	8	342.3	5.13	2.60	5.55	13.28	4.56	2.75	5.12
2	1	349.9	5.41	2.71	5.80	13.92	4.88	2.84	5.42
2	2	326.9	4.59	2.61	5.54	12.74	4.17	2.74	5.20
2	3	307.3	4.23	2.60	5.19	12.02	3.75	2.81	4.76
2	4	291.6	3.33	2.51	4.40	10.24	3.28	2.83	4.36
2	5	267.1	2.54	2.43	2.98	7.95	2.76	2.81	3.41

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[1] The basic amino acids were expressed as g/16g N.

[2] See Tables 17 and 18 for RSMs used in trials.

Table 26

Summary of Results of Growth Trial 2[1]

Ration[2]	Autoclave treatment, hr at 121°C	DBCP, mg dye/g protein	Weight gain, g/group	Feed intake, g/group	Crude[3] protein g/group	TPE[4]
1	0	349.9	3599	8049	1448	2.48
2	1/2	326.9	2959	7987	1438	2.06
3	1	307.3	2549	7507	1351	1.89
4	2	291.6	1366	7177	1292	1.06
5	4	267.1	618	6085	1095	0.57
S.E.M.[5]			±65	±202	±36	±0.05
D.F.[6]			10	10	10	10

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[1] The means of weight gain, feed intake, crude protein eaten and TPE were averages of three replicate groups.

[2] The same amount of RSM was incorporated into each ration (30.73%), see Table 18.

[3] The amount of crude protein eaten was calculated on the basis of 18% crude protein in the ration.

[4] The means were significantly different from each other ($P < 0.05$).

[5] Standard error of the means.

[6] Degree of freedom.

Thus, differences in the protein quality of commercial RSMs and deliberately heat-damaged RSMs have been clearly demonstrated. Rapeseed meals of commercial origin, despite their difference in ability to bind Acid Orange 12, were not measurably different in protein quality whereas 30 minutes of heating RSM in an autoclave at 121°C not only reduced its ability to bind Acid Orange 12 but had an adverse effect on chick growth. A comparison of the basic amino acid contents of the heat-treated RSMs and the commercial meals showed that the lysine content in the heat-treated Tower RSM was reduced by heating for as short a time as 30 minutes, from 5.41 to 4.59 g/16g N and further to 3.33 and 2.54 g/16g N after 2 and 4 hours of heating in the autoclave at 121°C. Of the other basic amino acids, arginine was affected to a similar degree to lysine, from 5.8 g/16g N in the untreated meal to 2.98 g/16g N after 4 hours of treatment. In the case of the meal heated for only 30 minutes, the lysine level of 4.59 g/16g N was lower than that found in any of the commercial meals.

The lower lysine and arginine contents of the autoclaved RSM were also reflected in the basic amino acid composition of the test rations (Table 25). The lysine levels of the rations (4.17 to 2.76 g/16g N) containing autoclaved Tower RSMs were all below the lysine levels of the rations (4.53 to 5.02 g/16g N) containing the RSM that was not autoclaved or the commercial

RSMs. Similar decreases in arginine but not in histidine were noted in the rations containing the meals heated in the autoclave from 1 to 4 hours.

It appeared that only RSMs with DBCP equal to or less than 326.9 mg dye/g protein were sufficiently inferior in the protein quality to permit detection biologically. Nevertheless, the overall findings from the feeding trials in the present study were in favour of the use of the dye-binding technique coupled with macro-Kjeldahl protein determination as a laboratory means of predicting the protein quality of RSMs, particularly of meals that may have been heat-damaged.

In the discussion above, the lack of difference in protein quality among the commercial RSMs but apparent among the autoclaved Tower meals were explained by the differences in dietary lysine levels in the rations. The presence of glucosinolates which are potentially growth depressing in the meals themselves was not considered. In this respect, the level of glucosinolates in the RSMs was more important in the interpretation of the TPE data obtained from Trial 1 where commercial high glucosinolate type RSMs were used. The exact glucosinolate contents of these meals were not known. This was further confounded by the fact that the percent of RSMs incorporated into the eight rations varied according to the protein percent of the meals (Table 17). Therefore, it was not known to what extent variation in the glucosinolate content of the rations affected the growth performance of the chicks. On the other hand, the influence of glucosino-

lates on the chick growth and on TPE was less important in the second trial since the RSM used was from Tower rapeseed known to contain a glucosinolate level approximately one-tenth of that found in high glucosinolate rapeseed (Stefansson and Kondra, 1975). Secondly, the five Tower meals, although autoclaved for various lengths of time, were derived from the same batch of RSM and were incorporated into the rations at the same level. The unknown levels of glucosinolates in the commercial RSMs might therefore be partially responsible for the poor agreement between the protein quality predicted chemically by the dye-binding method and the TPE measured in chick assay.

Studies on the nutritional quality of RSM reported to date have been aimed at establishing the optimal replacing levels of RSM for soybean meal in practical poultry feedings. Few studies have been undertaken to compare the protein quality of different RSMs. In an earlier study, Hill (1974) compared the nutritional quality of RSMs prepared by different oil extraction processes against soybean meal. Using a modified TPE method, RSM samples were used to supply 11% of the 17% protein in the test rations and the results showed that four of the nine RSMs evaluated gave TPE values significantly lower than the control soybean meal ration. These RSMs were not assayed for protein quality by any chemical means. The inferior protein quality of these RSMs could not be explained by their chemical composition or by glucosinolate contents.

The lack of correlation between the protein quality index as obtained by the dye-binding technique and by the TPE method for the commercial RSMs has been discussed. On the other hand, the highly positive correlation recorded for the laboratory heat-damaged Tower RSMs was in agreement with those reported for soybean meals by Moran _et_ _al_. (1963) and for barley by Mossberg (1966).

4 Summary

Two chick growth trials were undertaken to investigate the relationship between the protein quality index of commercial RSMs and laboratory heat-damaged RSMs as predicted chemically by the dye-binding method and biologically by the total protein efficiency (TPE) method. Results obtained from the study indicated that commercial RSMs which bound Acid Orange 12 from 335 to 360 mg/g protein were not significantly different in protein quality as shown in the chicks growth assay. However, the dye-binding technique was able to distinguish RSMs of inferior protein quality caused by heat particularly those heated in the autoclave for more than 30 minutes. RSMs with DBCP equal to 327 mg Acid Orange 12/g protein or less were of lower protein quality as shown by chick growth assay. The TPE values recorded from RSMs heated for various lengths of time ranked in the same order as the DBCP and were a function of heating time.

e Experiment 5: The Effects of Heating Laboratory Prepared Rapeseed Meal in an Autoclave or an Oven for Varying Periods of Time on Protein Denaturation in the Rapeseed and the Feasibility of the Dye-Binding Method for Measuring Such Denaturation

1 Objectives

The last of this series of dye-binding studies was to investigate the use of the dye-binding technique as a measure of protein denaturation in RSMs caused by various autoclave or oven heat treatments.

2 Materials and Methods

Raw rapeseed (B. campestris var. Span) was crushed in a laboratory blender with dry ice and its oil was extracted with hexane for 24 to 30 hours in a Soxhlet type extractor. The RSM prepared was then freeze-dried under vacuum to remove the water in the meal. The moisture content was reduced to 2% by this procedure.

The freeze-dried RSM was divided into several equal portions and distilled water was added to bring the moisture contents of the meals to 10, 20, 30 and 40%. The samples of RSM with the different moisture contents including the 2% freeze-dried meal were further subdivided into five portions and transferred to an autoclave or an oven preheated to 121°C. The RSMs were heated for 15, 30, 60, 120 and 240 minutes. After the heat treatments, the samples were frozen in a freezer at -20°C and those meals with moisture levels before heating above 10% were again freeze-dried to remove the excess water. However, the freeze-drying in this instance

was terminated when the moisture contents of the meals were reduced to 8 to 9%. The RSMs were reground to 40 mesh and stored in dessicators for chemical analyses.

The crude protein contents, moisture contents and DBCs with Acid Orange 12 of the RSMs were analysed as described in previous experiments. Available lysine contents of the RSMs were also determined.

The degrees of protein denaturation in the RSMs resulting from the two types of heat treatment for different periods of time were estimated by nitrogen solubility of the heated meals in water as follows: 2 g of RSM reground to a particle size of 60 mesh was weighed and transferred to a 250 ml conical flask. One hundred ml of distilled water was added to the flask and the meal was dispersed thoroughly with a stirring rod. The flask was sealed with parafilm and placed on a mechanical shaker. The mixture was shaken for 2 hours at the rate of 160 strokes per minute. The flask with its contents was allowed to stand for a few minutes and 60 ml of the solution was decanted into a centrifuge tube. The tube was centrifuged for 20 minutes at 2500 rpm. Two 25 ml aliquotes of the supernatant were assayed for soluble nitrogen by the Kjeldahl procedure (A.O.A.C., 1970). The amount of soluble nitrogen was expressed as percent of the total nitrogen in the RSM.

3 Results and Discussion

The degree of protein denaturation in the heated RSMs as measured by their nitrogen solubility (NS) is presented in Table 27 and shown graphically in Fig. 6. The data for

Table 27

Effect of Heating Rapeseed Meal in an Autoclave or Oven for Various Periods of Time on the Solubility of the Nitrogen of the Meals in Water[1,2]

Moisture, %	Form of heat[3]	Duration of heating, minutes				
		15	30	60	120	240
2	A	18.5	17.8	10.8	9.4	8.3
	O	34.0	29.6	12.2	5.5	5.5
10	A	16.8	12.7	7.3	8.6	11.5
	O	24.3	19.4	14.4	7.1	7.6
20	A	14.8	7.7	7.4	9.0	12.3
	O	23.1	21.8	13.6	8.0	7.4
30	A	8.2	8.4	8.7	10.7	14.2
	O	24.5	16.5	11.6	8.7	6.9
40	A	8.4	8.9	9.7	11.4	15.2
	O	25.6	14.1	11.4	9.1	8.7

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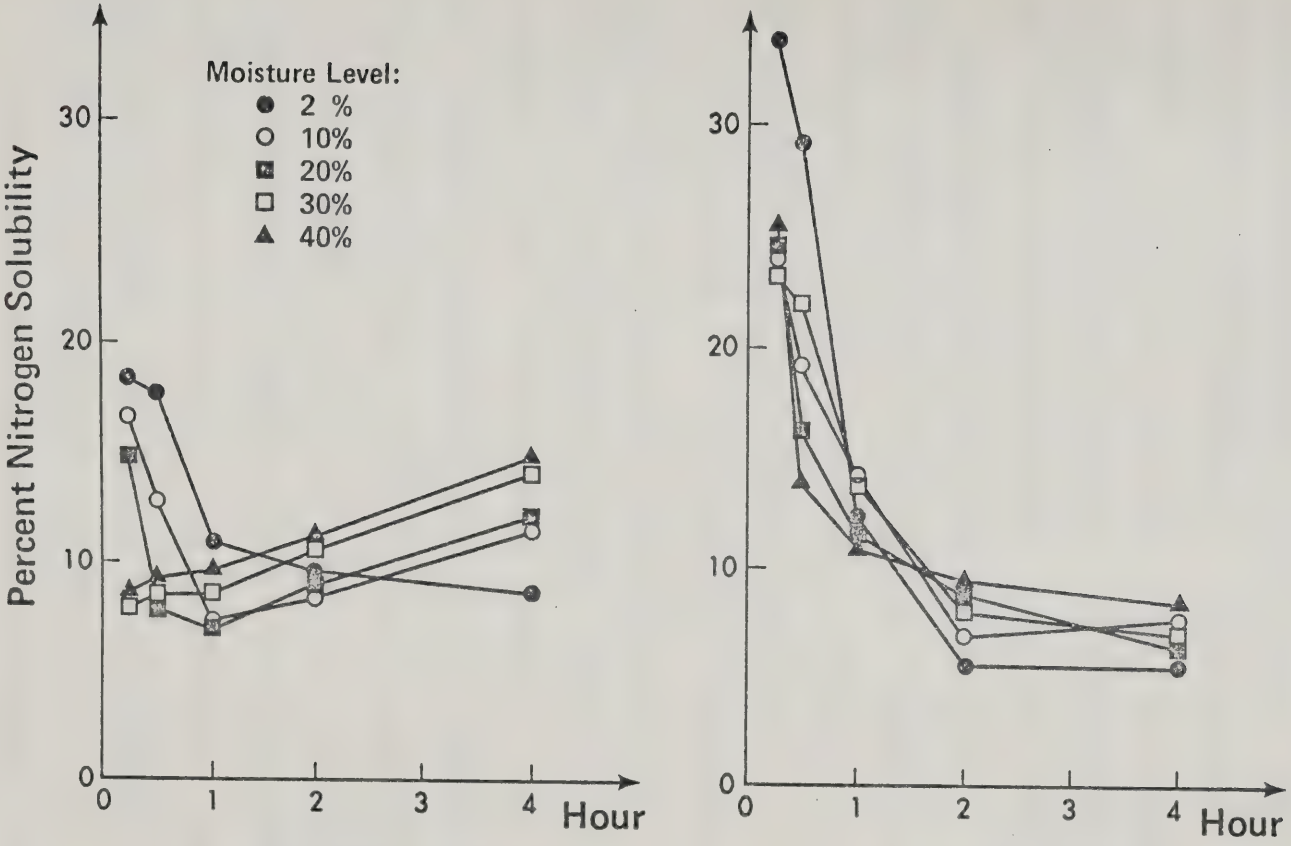
[1] The temperature of heat treatment was 121°C.

[2] The nitrogen solubility of the defatted RSM without treatment was 33.6%.

[3] A = autoclave; O = oven.



(a) Autoclave Heating

(b) Oven Heating

Fig. 6 Solubility of nitrogen as a function of time rapeseed meals heat-denatured in autoclave or oven at 121°C for various periods of time

NS were analysed statistically by a multiway analysis of variance. The results indicated that the NS of the RSMs was significantly affected by the three factors studied, namely, the form of heat applied to the meals, the moisture level in the meals and the duration of heat treatment. The treatment effects on the NS of the RSMs could be summarized as follows: decreases in NS were noted in all heated meals except the RSM which was oven-heated for only 15 minutes; when compared to meals which were oven-heated for 1 hr or less, meals which were autoclaved for 1 hour or less showed a greater reduction of NS but the effects were reversed when the heating duration was extended to 2 and 4 hours (Fig. 6).

If one used NS as a measure of denaturation it would appear from the data that some denaturation occurred with as little as 15 minutes of heat treatment in the autoclave whereas it took 30 minutes of heating in the oven to reduce NS. Protein denaturation was at its maximum when the RSMs were autoclaved for 1 hour or oven heated for 2 hours. The level of moisture present in the meals also affected the extent of protein denaturation. When the denaturation of heat treatment was short, the presence of high moisture aggravated protein denaturation but appeared beneficial when treatment duration was prolonged.

In addition to recorded NS, changes in color of the meals as a result of the heat treatments were noted. A brownish color began to develop in RSMs autoclaved for as short a time as 15 minutes and in meals heated in the oven

for 30 minutes. The color development intensified in direct relationship to heating time. Intensity of color development has been related to the degree of protein denaturation in soybean meal during heat treatment by Beckel *et al*. (1942).

To study the effects of the three factors on the dye-binding capacity of the protein (DBCP) of the RSMs, the crude protein and DBC of the meals were determined and the DBCP with Acid Orange 12 were calculated as in previous experiments. The data are tabulated in Table 28 and shown graphically in Fig. 7. In order to examine the relationship between the degree of protein denaturation and the corresponding binding ability of the RSM protein with Acid Orange 12, the correlation between the DBCP data and NS was determined ($r = 0.63$). Although the correlation obtained was highly significant ($P<0.01$), examination of the graphs in Figures 6 and 7 showed that there were some differences in the degree of protein denaturation as measured by NS and by DBCP for the same RSM samples. From the two figures shown, the rates of decrease in DBCP and of NS for RSMs differed for meal subjected to the same heat treatment. The DBCP of autoclaved RSMs showed little decrease in the first 15 minutes of heating but declined sharply thereafter until 2 hours of heat treatment was reached. Continued heating in the autoclave did not further reduce the DBCP of the meals. This observation was in direct contrast to the NS of the same autoclaved RSMs in which a reverse in NS were noted when the heating duration was longer than 2 hours. For the oven-heated RSMs, the rate

Table 28

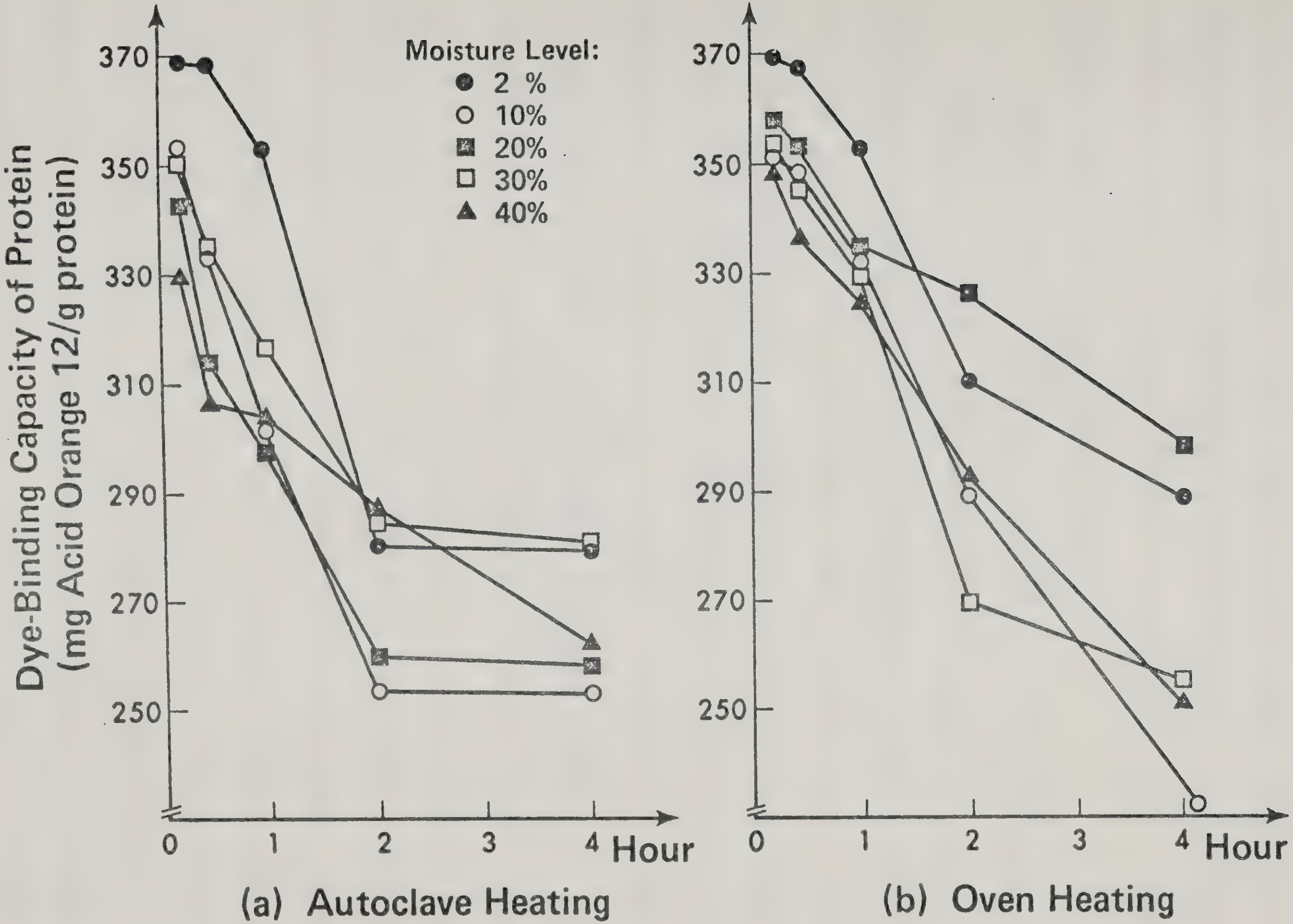
Dye-Binding Capacities of the Protein of Rapeseed Meal Heated in an Autoclave or Oven for Various Periods of Time[1,2]

Moisture, %	Form of heat[3]	Duration of heating, minutes				
		15	30	60	120	240
2	A	368.5	367.0	353.1	282.6	279.5
	O	369.2	367.2	352.7	310.1	289.2
10	A	352.8	333.2	302.5	253.5	253.5
	O	352.2	346.9	331.5	268.4	253.8
20	A	342.4	312.7	297.1	258.9	257.3
	O	350.4	342.0	329.4	290.6	228.5
30	A	329.5	307.5	304.3	286.6	261.8
	O	348.8	335.0	323.6	291.2	251.0
40	A	351.6	334.2	315.4	305.1	280.6
	O	357.2	350.5	332.4	325.1	296.5

[1] The temperature of heat treatment was 121°C.

[2] Unit of DBCP was mg Acid Orange 12 per g protein.

[3] A = autoclave; O = oven.



(a) Autoclave Heating

(b) Oven Heating

Fig. 7 Dye-binding capacities of protein of rapeseed meals heated in an autoclave or oven at 121°C for various periods of time

of decrease in DBCP was slow in the RSMs heated for 15 minutes to 1 hour but was rapid from then until the lowest DBCP was reached at the end of 4 hours of heating. Statistical analysis of the DBCP data showed that the ability of the RSM protein to bind Acid Orange 12 was significantly affected by the three factors studied, the length of heating being the most important one.

The available lysine contents of the heat treated meals expressed as mg/g protein are presented in Table 29. The determinations were undertaken mainly to estimate the nutritive values of the heat-treated RSMs and to make comparisons between nutritive and DBCP values. The tabulated available lysine data have been adjusted for the loss of DNP-lysine during the analytical procedure. Similar to the observed decreases in DBCP, available lysine levels in the RSMs were significantly reduced by increasing the time of heat treatment ($P<0.01$). The rate of decreases in available lysine was dependent mainly on the duration of heat treatment. However, oven-heating appeared to be less detrimental than autoclaving to available lysine content. On the other hand, it was noted that high levels of moisture in the RSMs decreased the adverse effects of heat treatment on the available lysine contents of the meals. The correlation coefficient ($r = 0.90$) between the DBCP and the available lysine of the RSMs was highly significant ($P<0.01$).

Protein denaturation has been defined by Neurath et al. (1944) as "any non-proteolytic modification of the unique

Table 29

Available Lysine Contents of Rapeseed Meals Heated in an Autoclave or Oven for Various Periods of Time[1,2]

Moisture, %	Form of heat[3]	Duration of heating, minutes				
		15	30	60	120	240
2	A	54.3	53.6	43.7	24.3	8.4
	O	58.9	59.4	51.0	14.0	7.3
10	A	50.6	49.1	26.2	18.4	13.8
	O	51.3	56.3	45.1	13.3	9.7
20	A	54.6	47.1	37.5	24.5	13.2
	O	50.3	48.4	43.7	29.5	6.3
30	A	49.5	48.3	36.8	24.6	16.5
	O	55.3	55.0	51.3	41.5	17.1
40	A	44.8	42.5	37.1	30.1	20.4
	O	56.5	49.3	42.5	40.8	22.2

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[1] Available lysine content was expressed as mg per g protein.

[2] Correlation coefficient between available lysine and DBCP was 0.90 and was highly significant ($P<0.01$).

[3] A = autoclave; O = oven.

structure of native proteins, giving rise to definite changes in chemical, physical or biological properties". Although several factors including heat, chemicals and pH are known to denature protein, the most likely factor involved in the denaturization of protein in oilseed meals during processing is the exposure to excess heat treatment.

Interest in studying protein denaturation in oilseed meals arose mainly from attempts to establish optimal processing conditions for the production of isolated protein and to maintain uniform processing operations for optimal nutritive value. Reports on protein denaturation in soybean meal during processing or in the industrial production of isolated soybean protein appeared in the literature as early as 1938 (Smith and Circle, 1938; Beckel et al., 1942; Pomeranz and Lindner, 1960).

Rutkowski et al. (1966) were the first to investigate the effects of processing conditions on protein denaturation in RSM. In a study involving steam heating of RSM at 105° to 135°C for 30, 60 and 90 minutes, they found that the least decrease in soluble nitrogen occurred in meal heated at 105°C for 30 minutes. Increasing temperature and particularly time of processing decreased soluble nitrogen.

The results of the current study involving RSM containing a normal level of moisture (10%) agree well with those reported by Rutkowski et al. (1966). Although the autoclave treatment was more severe in our study, the greatest amount of soluble nitrogen was found in the RSM receiving the

mildest heat treatment (15 minutes) and decreased progressively as the time of heating increased to 1 hour. The NS of the RSMs having moisture contents of 2 to 20% and autoclaved for only 15 minutes also agreed well with values reported by Finlayson (1966) and Lo and Hill (1972). The former worker found that water dissolved about 20% of the meal nitrogen while the latter group of workers reported that between 14-18% of the original meal nitrogen was water soluble.

The effects of dry and moist heats on rapeseed protein denaturation were examined by Girault (1973). Laboratory defatted rapeseed flour and industrial RSM were heated at 105°C on a stove either dry or with 8% moisture for 30 minutes to 3 hours. It was found that heating in the presence of moisture produced harsher effects on nitrogen solubility in both alkaline and saline solutions than heating in the absence of moisture. Similar observations on soybean meal protein denaturation by moist and dry heats at various temperatures were reported by Beckel *et al*. (1942) and on defatted soy flakes by Pour El (1973). In the present study, more damaging effects of the autoclave treatments compared to those from the oven heating treatments on the solubility of nitrogen in RSMs were evident from 15 minutes to 1 hour of heating but were reversed from 2 to 4 hours.

From the results of the present study, the changes in DBCP of the RSMs as a result of the heat treatments did not agree with the decreases in NS of the meals subjected to the same treatments. Thus, for the same RSM, the extent of protein denaturation measured by the two methods might

be expected to differ considerably. This difference probably arises from the fact that the two determinations measure different properties of the heat-denatured RSMs. While the NS determinations measured changes in physicochemical properties, the DBCP determinations estimated the changes in total basic groups in the protein and consequently the nutritive values of the protein. Furthermore, the NS values for the RSMs include non-protein nitrogen as well as protein nitrogen and therefore should not be as reliable as index of the nutritional value of the RSM as the DBCP of the RSM. The higher correlation coefficient between DBCP and available lysine, than between DBCP and NS (0.90 versus 0.63) also suggests suggests that the DBCP of the RSM is a better index of heat damage when nutritional quality is the prime consideration of acceptability of the RSMs. However, in food technology where functional and physical properties of isolated oilseed protein, rather than its nutritive value, will largely determine its acceptability as an ingredient in prepared foods, the method of nitrogen solubility determination is preferable.

4 Summary

Laboratory produced RSMs with moisture contents ranging from 2 to 40% of the meal were heated in an autoclave or an oven at 121°C from 15 minutes to 4 hours. The degree of protein denaturation of the RSMs was estimated by nitrogen solubility in water (NS) and by dye-binding with Acid Orange 12. Results of the study indicate that heating RSM for 15 minutes to 1 hour in an autoclave produced more severe

denaturation than heating RSM at a similar temperature for the same periods of time in an oven. When heating was prolonged the denaturation trends were reversed. The ability of the RSM protein to bind Acid Orange 12, calculated as the dye-binding capacity of the protein (DBCP), was more severely affected by the autoclave-heating treatments than by the oven-heating treatments. In this regard, the DBCPs of the protein-denatured RSMs were at the lowest after 2 hours of autoclave-heating and after 4 hours of oven-heating.

Since the NS values for the RSMs include non- protein nitrogen, while DBCP values measure mainly basic amino acids, the latter values are likely to be more valid for estimating the nutritional value of RSM. The higher correlation coefficient that was found in this study between DBCP and available lysine than that between NS and available lysine (0.90 versus 0.63 respectively) would seem to support this view.

PART II

EFFECT OF THE LEVELS OF THE GLUCOSINOLATES IN RAPESEED MEAL ON THE TRANSFER OF DIETARY IODINE TO EGGS BY LAYING HENS

A Introduction

Most of the thyrotoxic properties of rapeseed meal (RSM) have been attributed to the presence of glucosinolates in the meal. During the course of passage of meal through the gut of animals, myrosinase-like enzymes present in the gut hydrolyse these compounds to produce substances which impair thyroid function and cause enlargement of the glands (Greer and Deeney, 1959). One of the most studied glucosinolates found in rapeseed is progoitrin (2-hydroxy-3-butenylglucosinolate). This compound on hydrolysis yields glucose, bisulfate and 2-hydroxy-3-butenyl isothiocyanate (Greer, 1956) which is unstable and cyclizes to form goitrin, (-)-5-vinyl-2-oxazolidinethione (Kjaer, 1960). Goitrin has been shown in studies with growing chickens to interfere with the power of the thyroid glands to synthesize thyroxine (Matsumoto *et al*., 1968).

Until recently the use of RSM in rations for monogastric animals and poultry has been limited by its high content of glucosinolates. This has been particularly true with respect to RSM derived from *Brassica napus* type seed which contains a higher level of glucosinolates than RSM derived from *Brassica campestris* type seed (Clandinin *et al*., 1972). However, RSM derived from rapeseed that contains a very low level of glucosinolates has become available recently for feeding to

livestock and poultry. The objective of the study described herein was to compare the effects of including high or low glucosinolate type RSM (HG-RSM or LG-RSM) at various levels in the ration of laying hens on the uptake of iodine by the thyroid glands of layers fed rations containing such meals and on the transfer of iodine to eggs.

B Review of Literature

Despite the known goitrogenic properties of RSM and their effects on the general health and well-being of domestic animals (Rutkowski, 1971; Rapeseed Association of Canada, 1972), the transfer of goitrogenic substances in RSM to milk, meat and eggs has been the object of only limited studies.

Earlier investigations on the transfer of goitrogenic compounds to milk as a result of feeding dairy cows with crystalline oxazolidinethione and thiocyanate in quantities similar to amounts fed as green rape fodder indicated that these compounds seemed to be transferred to milk (Virtanen et al., 1958, 1959; Virtanen and Gmelin, 1960). The amounts found in milk were so small that it seemed unlikely that they could cause disturbances in the functioning of the thyroid glands of man consuming such milk. A considerable decrease in the iodine content of milk was, however, noted (Piironen and Virtanen, 1963). A reduction in the iodine content of milk from cows fed RSM was also reported by Iwarsson (1973). Cows were fed protein concentrates containing 0, 4.2, and 8.05% RSM according to milk yields. A significantly lower iodine concentration in milk from the RSM fed

groups compared to that of the control group was noted after five weeks of lactation. Based on the estimation of the iodine intake per day, the mean percents of iodine secreted in the milk calculated over the whole lactation were 0.72% in the group given 8.05% RSM and 1.2% in the control group. A higher thiocyanate concentration in milk produced by cows fed RSM as compared to those fed no RSM was also noted. In a related study, milk produced by the three groups of cows were further examined for their goitrogenic properties in a rat assay over a five week test period (Iwarsson and Nilsson, 1973). Rats fed milk from cows given concentrates with 4.2 and 8.05% RSM showed thyroid hypertrophy and lowered serum protein bound iodine (PBI). Uptake of a single dose of ^{131}I was higher in the RSM groups than in the control group. These changes were attributed to the lower iodine content of the milk.

In poultry, goitrogenic effects on the progeny of breeding hens fed RSM were first reported by March et al. (1972). Hypertrophy of the thyroid glands and reduced body weight of day-old chicks hatched from eggs produced by hens fed rations containing high levels of RSM over a prolonged period of time were reported. Since the type of RSM used contained a considerable amount of glucosinolate, it was first thought that one or more of the antithyroid substances resulting from glucosinolate hydrolysis might have been transferred to the eggs and thus resulted in thyroid enlargement of the progeny. However, extensive studies by Roos and Clandinin (1975) failed

to detect the presence of hydrolysis products of glucosinolates in eggs from layers fed rations containing RSM. This led these workers to investigate an alternate hypothesis, namely, that the thyroid hypertrophy noted in day-old chick was the result of a decreased iodine content in eggs from breeders fed RSM. They found that transfer of ^{125}I to egg yolk was affected by the presence of RSM in the ration of the laying hens and the levels of RSM included in the ration. The effect was more severe when a source of myrosinase, the enzyme responsible for the release of goitrin from the progoitrin in RSM, was also included in the ration.

C Experiment Conducted at The University of Alberta

a Materials and Methods

Fifty Single Comb White Leghorn (SCWL) layers that had been laying for six months were selected for the study. They were placed in individual cages and fed a control ration with soybean meal as the main source of supplementary protein. The control ration contained 0.3 mg/kg diet of added iodine supplied by potassium iodate. After the birds were on the control ration for two weeks, eggs produced over a three day period were collected for iodine analyses. The fifty birds were then divided into ten groups of five birds each and duplicate groups were assigned to each of the experimental rations (Table 30). The rations consisted of a control ration and four RSM rations which contained either HG-RSM or LG-RSM included at the 5 or 10% level. Water and feed were supplied ad libitum. After the birds had been on the experimental rations for six weeks a further three day collection of eggs was made for iodine analyses.

Table 30

Composition of Experimental Rations

Ingredients	Rations 1	2	3	4	5
Basal[1]	80.00	80.00	80.00	80.00	80.00
Ground wheat	12.00	8.40	4.80	8.90	5.80
Soybean meal (48% protein)	7.00	4.30	1.60	3.85	0.70
HG-RSM[2]	-	5.00	10.00	-	-
LG-RSM[3]	-	-	-	5.00	10.00
Stabilized fat	1.00	2.30	3.60	2.25	3.50
KIO_3[4]	+	+	+	+	+

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[1] Contained per 80 kg; ground wheat, 41.8; ground barley, 10.0; ground oats, 10.0; wheat shorts, 1.12; dehydrated alfalfa meal, 2.0; soybean meal, 5.2; ground limestone, 7.0; calcium phosphate, 1.5; NaCl (CP), 0.5; MnO, 0.025; ZnO, 0.01; 50% choline chloride, 0.02; vitamin AD premix, 0.5; vitamin B premix, 0.3; vitamin E premix, 0.025.

Vitamin AD premix supplied 6,000 IU of vitamin A and 1,200 IU of vitamin D_3 per kg of complete ration. Vitamin B premix supplied 3 mg of riboflavin, 6 mg of calcium pantothenate, 15 mg of niacin and 7.5 ug of vitamin B_{12} per kg of complete ration. Vitamin E supplied 11 IU per kg of complete ration.

[2] High glucosinolate rapeseed meal, mixed B. napus varieties of low erucic acid type.

[3] Low glucosinolate rapeseed meal (B. napus var. Tower).

[4] Added at rate of 0.5 mg per kg of ration.

Eight birds, selected according to their egg production records, were drawn from the birds receiving each treatment ration for study of ^{125}I transfer to egg yolk. One ml of 0.9% saline containing 10 μC of ^{125}I was administered daily for eleven days, by lowering a pipette directly into the crop of the test bird. The ^{125}I solution contained 0.4% of 0.02N NaOH to prevent volatilization of the iodine.

Counting of ^{125}I activity incorporated in egg yolk and egg albumen began on the day following ^{125}I oral administration and was carried out as follows: the eggs were weighed, broken and separated into yolk and albumen and their weights were recorded separately. Egg yolk and egg albumen fractions were homogenized and duplicate samples (approximately one gram) of each were transferred to counting vials. The activities of ^{125}I in yolk and albumen samples were counted using a Biogamma Counter. Standards were prepared by diluting the daily dose with distilled water to volumes similar to those for the yolk and albumen and were counted as referred to above. The amounts of ^{125}I transferred to yolk and albumen were expressed as percentages of the daily dose received by the test bird.

At the end of the experiment, the birds were killed and the thyroid glands were removed and weighed. Approximately 20 mg of the thyroid glands of each birds was homogenized with 10 ml of distilled water. Duplicate 1 ml samples of the homogenates were transferred to counting vials and counted. The total activity of ^{125}I taken up by the thyroid glands was

expressed as a percentage of the weekly dose received by the test birds.

Samples of eggs collected prior to administration of radioactive iodine were broken and separated into yolk and albumen. The two fractions were then freeze-dried and stored in a freezer at -13°C for chemical determination of their iodine contents according to the procedure of Foss *et al.* (1960). The amount of iodine was expressed in ug I/g dry yolk and albumen.

The potential isothiocyanate and oxazolidinethione contents of the RSMs were determined according to the method of Appelqvist and Josefsson (1967).

Data obtained in this experiment were subjected to statistical analysis by a one-way analysis of variance. Two of the test birds did not lay during the oral administration of ^{125}I and thus resulted in unequal observations among treatments on the percentages of ^{125}I incorporated into egg yolk and in thyroid. Means which were significantly different as indicated by the F test were further analysed by Duncan's Multiple Range test (Steel and Torrie, 1960).

b Results and Discussion

Analysis of the RSMs used showed that the HG-RSM contained 2.5 mg/g meal of potential isothiocyanates and 6.4 mg/g meal of potential oxazolidinethione whereas the LG-RSM contained potentially 0.6 and 0.4 mg/g meal, respectively, of these two hydrolytic products. Thus, the glucosinolate content of the HG-RSM was approximately 10 times that of the LG-RSM.

The antithyroid effects of the RSMs are shown in Table 31. Statistical analysis of the means for thyroid gland size in mg/100 g body weight for the various treatment groups showed that the thyroid gland sizes of the birds fed the HG-RSM - containing rations (rations 2 and 3) were significantly heavier than those fed the LG-RSM-containing rations or the control ration.

Fig. 8 shows the rates of accumulation of ^{125}I in egg yolk in percent of daily dose by laying birds fed the five experimental rations. The means used in plotting the curves were the averages of the percentages for ^{125}I incorporated in the yolk fraction of eggs laid daily by birds receiving the same ration. The number of eggs laid per group varied from two to eight per day. It will be noted that the ^{125}I content of egg yolk has reached a plateau by the seventh day. Once the steady state was reached. the amount of ^{125}I transferred to egg yolk remained fairly constant. The small daily variations in the means within treatments appeared to be caused by the variation in number of eggs laid daily. The activity of ^{125}I found in egg albumen was higher than that of egg yolk on the first and second days after radioiodine administration. However, by the time the steady state was reached, the activity in egg albumen had become very slight, less than 1% of the daily dose , and, as a consequence, the data are not reported here. The plateaus obtained for the five treatment groups during the steady state clearly indicated a trend for less ^{125}I being trans-

Table 31

Percent ^{125}I in Egg Yolk During Steady State, ^{125}I Uptake by Thyroid Glands and Thyroid Size of Hens

Treatments[1]	^{125}I in egg yolk in steady state, expressed as % of daily dose	^{125}I in thyroid glands, expressed as % of weekly dose	Thyroid weight, mg/100 g body weight
Control	13.3^{a2}	12.6^{a}	7.6^{a}
HG-RSM - 5%	10.1^{bc}	25.8^{b}	12.5^{b}
HG-RSM - 10%	8.1^{c}	34.7^{b}	19.6^{b}
LG-RSM - 5%	14.2^{a}	16.2^{a}	7.9^{a}
LG-RSM - 10%	12.3^{ab}	18.2^{a}	7.8^{a}
S.E.[3]	±0.9	±2.1	±2.1

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[1] See Table 30 for description of meals.

[2] Means within column with the same superscript were not significantly different ($P<0.05$).

[3] Standard error of the mean with n=33.

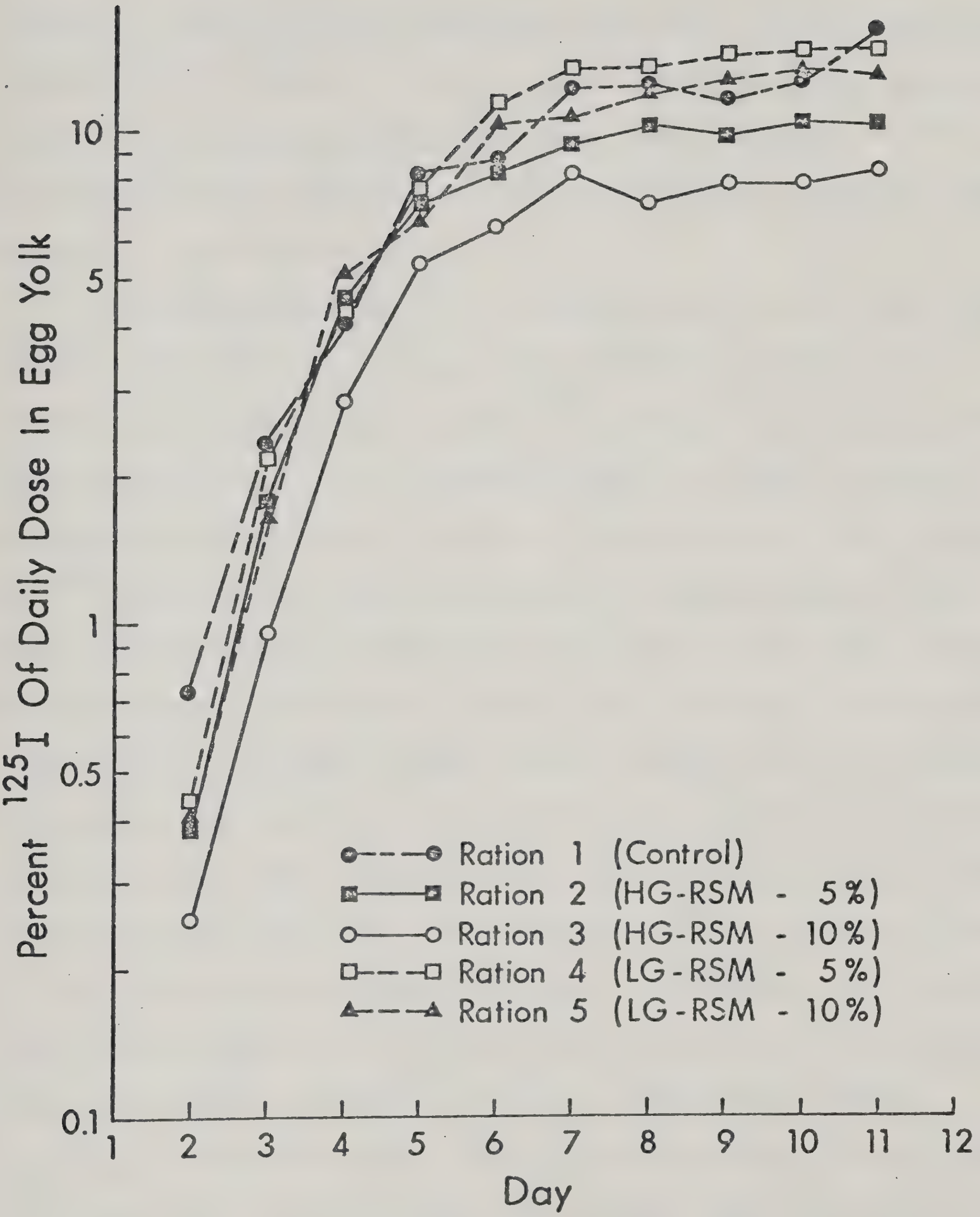

Fig. 8 Rate of ^{125}I incorporation in egg yolk

ferred to egg yolk when the birds were fed rations containing the HG-RSM than when the birds were fed the control ration or the rations containing LG-RSM. The least transfer of ^{125}I was found in the egg yolk of the eggs laid by birds fed 10% HG-RSM in their ration.

The percentages of ^{125}I of daily dose in egg yolk between the seventh and eleventh days for individual birds were averaged and considered as the ^{125}I transfer to egg yolk during the steady state. The treatment means are shown in Table 31. Statistical analysis of the data indicated that the transfer of ^{125}I to egg yolk was significantly reduced when HG-RSM was included in the laying rations at both 5 and 10% levels as compared to the control ration. On the other hand, the amounts of ^{125}I found in egg yolk of layers fed the LG-RSM-containing rations were not significantly different from the control group. The adverse effect of glucosinolate in RSM on the transfer of orally administered ^{125}I was most severe in the group receiving the 10% level of HG-RSM in its ration. The results obtained in the present experiment are in agreement with those reported by Roos and Clandinin (1975). However, the means of 10.1 and 8.1% for the two groups fed the 5 and 10% HG-RSM rations were higher than those reported by Roos and Clandinin (1975) for birds fed corresponding levels of RSM with 3% of ground raw rapeseed to provide the enzyme (myrosinase) necessary for the hydrolysis of the glucosinolates present in the RSM. Thus, in the absence of an exogeneous source of the enzyme the effect of glucosino-

lates on ^{125}I transfer is less severe. It was also noted that the percentage of ^{125}I found in egg yolk was inversely related to the degree of thyroid gland hypertrophy.

The amounts of ^{125}I taken up by the thyroid glands of the birds are also presented in Table 31. The activity of ^{125}I was expressed as percent of weekly dosage. Statistical analysis of the data revealed that the percentages of ^{125}I uptakes by the thyroid glands were significantly higher for the laying hens fed the HG-RSM containing rations than for the other groups. The birds fed rations 2 and 3 retained in their thyroid glands 25.8 and 34.7% ^{125}I of the weekly dosage, about two and three times, respectively, that of the control group. For the birds fed rations 4 and 5 the uptakes were 16.2 and 18.2%, only slightly, but not significantly, higher than the control group. This observation agrees with those previously reported for growing chicks by Clandinin _et_ _al_. (1966), Matsumoto _et_ _al_. (1968, 1969) and Bergner and Schmide (1972) using synthetic goitrin, (-)-5-vinyl-2-oxazolidinethione. In all cases, increased ^{131}I uptake and reduced release of ^{131}I were noted. In the present study, in spite of the increased thyroid uptake of ^{125}I noted in the thyroid hypertrophied birds, less ^{125}I was transferred into egg yolk.

The iodine content of egg yolk from eggs produced at the end of the pre-experimental and experimental periods are shown in Table 32. The iodine content of the egg yolk after the groups had been fed the control ration for two week ranged from 0.76 to 0.88 µg/g dry egg yolk and were not significantly

Table 32

Iodine Contents of Egg Yolk and Egg Albumen After Pre-Experimental and Experimental Periods

	After two week pre-experimental period	After six week experimental period	
Treatments[1]	ug I/g dry yolk	ug I/g dry yolk	ug I/g dry albumen
Control	0.76	0.84^{a}[2]	0.07
HG-RSM - 5%	0.80	0.52^{b}	0.06
HG-RSM - 10%	0.84	0.53^{b}	0.07
LG-RSM - 5%	0.79	0.70^{ab}	0.08
LG-RSM - 10%	0.88	0.65^{b}	0.07
S.E.[3]	±0.06	±0.06	±0.01

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[1] See Table 30 for description of meals.

[2] Means within column with the same or no superscript were not significantly different ($P<0.05$).

[3] Standard error of the mean with n=35.

different statistically. However, after the birds had received the RSM-containing rations the iodine levels in egg yolk were reduced. For the groups fed 5 and 10% HG-RSM-containing rations the means were both significantly different from that of the control group. Although the mean of the group fed the 10% LG-RSM-containing ration was also significantly different from the control, the decrease in iodine content was considerably less than for the HG-RSM treatments. This observation thus indicated that the effect of including LG-RSM in laying rations on the transfer of dietary iodine to eggs was less severe than when HG-RSM was included in laying rations. The slight decrease in dietary iodine transfer caused by the LG-RSM could perhaps be counteracted by increasing the level of supplementary iodine in the ration. The iodine contents of egg albumen are also presented in Table 32. There were no significant differences among the treatment means. Since the iodine content of egg albumen was not affected by the RSM in the rations, the albumen samples collected when all of the birds were on the control ration were not analysed.

Further studies should be conducted to establish the supplemental level of iodine in layer or breeder rations for the production of eggs with normal iodine content when RSM is included in the ration.

c Summary

The experiment was designed to evaluate the effect of the glucosinolate content of RSM on the transfer of dietary iodine to eggs. Five experimental rations involving two

levels (5 and 10%) of both high and low glucosinolate RSMs and a laying ration devoid of RSM were used in the study. The rations contained 0.3 mg of added iodine per kg diet and each ration was fed to two groups of five laying hens for six weeks before they were subjected to oral administration of ^{125}I daily for a period of eleven days.

Percent ^{125}I incorporated into egg yolk during the steady state which occurred seven days after administration of ^{125}I showed that the amount of ^{125}I transferred to egg yolk was significantly reduced by the inclusion of high glucosinolate RSM in the laying rations but not by the inclusion of low glucosinolate RSM. However, determination of the iodine content of egg yolk by chemical analysis indicated that there was some reduction in dietary iodine transfer to eggs even with the low glucosinolate RSM but the effect was less severe than that observed when high glucosinolate RSM was used.

PART III

STUDIES ON THE PROBLEM OF THE LAYING OF FISHY EGGS BY HENS FED RATIONS CONTAINING RAPESEED MEAL

A Introduction

In recent years, studies conducted in Europe and in Canada have revealed that layers of brown-shelled eggs fed rations containing rapeseed meal (RSM) produce significant numbers of odoriferous eggs. The odor was described as fishy.

Following the reports on the occurrence of the problem, studies were initiated to determine the breeds of layers which were most likely to produce fishy eggs when RSM was included in the ration and to identify the compound(s) which is responsible for the fishy taint. Attempts were also been made to determine the causative factor(s) or precursor in the RSM. The results of these studies are presented in the review below.

B Review of Literature

The earliest report on this problem was presented by Vogt *et al*. (1969). In a trial with laying hens, covering the period 20 to 70 weeks of age, a ration containing 20% of RSM was found to cause the laying of eggs that smelled like mustard oil. The level of RSM in the laying ration required to produce the tainted eggs was established as 8%. Although the factor(s) which caused the taint was not identified, these workers indicated that the potential content of (-)-5-vinyl-2-oxazolidinethione was high in the RSM used in the trial.

The problem was investigated in England by Hobson-Frohock _et_ _al_. (1973) following reports of the occurrence of tainted eggs in various parts of the United Kingdom (Miller _et_ _al_., 1972). In a study involving eggs from flocks of chickens in which laying of tainted eggs was reported, Hobson-Frohock _et_ _al_. (1973) identified trimethylamine (TMA) as the compound responsible for the fishy taint. The compound was found concentrated mainly in the yolk and became detectable above the level of 1 ppm. In the same study, it was found that when TMA·HCl (100 mg/day) was fed to the selected birds, characteristically fishy eggs containing 3 to 40 ppm of TMA were produced within six days. The same phenomenon was produced in these selected birds by feeding rations containing RSM. The meal was found to contain less than 1 ppm of TMA and thus was ruled out as the direct source of TMA found in the yolk.

In a follow-up study, Hobson-Frohock _et_ _al_. (1975) found that the causative factor(s) could be extracted from RSM by aqueous acetone. Selected birds, with previous records of producing tainted eggs, were fed a control ration and later changed to rations containing RSM, fat extract of RSM, acetone extract of RSM and the residue left after acetone extraction of RSM. A significant increase in the concentrations of TMA in the eggs laid by birds receiving the ration containing RSM or the acetone extract of RSM was found. Several samples of RSM from various sources were also tested and all were shown to cause the laying of eggs containing TMA. The RSMs were

further analysed for isothiocyanates, goitrin, nitriles, fatty acids and sinapine. None of these was found related to the level of TMA in the yolk.

According to Overfield and Elson (1975), RSM incorporated into laying ration at as low a level of 3% caused susceptible birds to lay tainted eggs. With 3, 6 and 9% RSM in the rations of brown-shelled egg layers, fed over a period of 28 days, fishy eggs produced were: 1.2, 19.3 and 20.9% of the total egg production, respectively. The first fishy egg was laid on the fifth day after the feeding of the rations containing RSM was commenced. The incidence of tainted egg production was at its peak during the second and third week of feeding the RSM-containing rations.

Comparison of feeding different RSMs to both white and brown-shelled egg layers on the incidence of fishy egg production was reported by Clandinin _et_ _al_. (1974). In this study, Span or Bronowski RSM fed at the levels of 5 and 10% in the rations to Single Comb White Leghorn (SCWL, white-shelled egg layers) did not affect the odor or taste of the eggs produced. However, inclusion of 6.8% of Span RSM in the ration of brown-shelled egg layers did cause the laying of eggs with fishy odor and flavour. Odor evaluation on raw eggs produced by Rhode Island Reds and White Plymouth Rocks (brown-shelled egg layers) showed that both breeds of layers may lay appreciable percentages of fishy eggs when fed rations containing RSM and that in the case of the RIRs the intensity of the fishy odor did not decrease with the length

of time the birds were on the RSM-containing ration.

Bolton et al. (1976) studied the possibility of eliminating tainted egg layers by genetical selection. They demonstrated that tainting of eggs by hens fed RSM-containing rations is conditional on the presence in the hen, in the heterozygous or homozygous state, of an autosomal semi-dominant gene that has variable expression, depending on environmental factors, including the level of RSM in the ration. Hobson-Frohock et al. (1975) have suggested that the tainting problem may be a consequence of certain hens being unable to metabolize TMA to TMA oxide as in the normal hen.

From the findings cited above, it is obvious that the factor(s) in RSM which causes the production of fishy eggs by brown-shelled egg layers remains unknown. While Vogt et al. (1969) implied that glucosinolates or their hydrolytic products appeared to be implicated in the production of fishy eggs, this was not confirmed by Clandinin et al. (1974) or by Overfield and Elson (1975).

In most of the above studies the RSM used, particularly the meals of European origin, were mainly from high glucosinolate varieties of rapeseed and little variation in ability to cause the laying of fishy eggs was noted among the RSMs. Since 1974, a new low glucosinolate variety of rapeseed (*B. napus* var. Tower) has become available for study. The glucosinolate content of this variety is only about one-tenth that found in RSMs previously studied (excluding the work on meal produced from Bronowski rapeseed which was fed only to white-

shelled egg layers). If glucosinolates are, in fact, not a factor in causing fishy eggs this should become evident when Tower RSM is included in the ration of brown-shelled egg layers. The first experiment described in this part of the thesis deals with such a study.

Another factor which, hitherto, has received little attention in the study of the laying of fishy eggs when RSM is included in the laying ration is the level of choline in RSM-containing rations. According to Klain et al. (1956), RSM contains approximately 7000 mg of choline per kg meal, which is twice that found in soybean meal. Choline (hydroxyethyl trimethylammonium hydroxide) is widely distributed in biological materials as free choline or as a moiety of phospholipids. It is characterised by a trimethyl quaternary nitrogen which breaks down to TMA when treated with alkali. Trimethylamine (TMA) formation from choline by the action of intestinal bacteria has been shown to occur in normal people or patients with hepatic diseases (De La Huerga and Popper, 1951). The TMA released is readily absorbed from the intestine and excreted in the urine mainly as TMA oxide. The microbial conversion of choline to TMA was confirmed by Prentiss et al. (1961) in a study involving the feeding of large amounts of choline chloride to rats raised under conventional and germ-free conditions. Choline chloride administered intragastrically to normal rats (200 mg/kg body weight) gave rise to urinary total TMA in yields of 10-66% of the administered dose. However, germ-free rats given the same dose of choline chloride

excreted only 0.6% of the choline as TMA. The action of intestinal microbes on choline was also shown to be depressed by simultaneous ingestion of antimicrobial agents such as aureomycin, terramycin and penicillin 0 (De La Huerga *et al.*, 1953).

In view of these findings on the formation of TMA from choline in the gut by microorganisms, it was thought that the high level of choline in RSM might be involved in the fishy egg problem. As a consequence, the second experiment was designed to study the possible relationship between choline content of the ration and the laying of fishy eggs by brown-shelled egg layers.

C Experiments Conducted at The University of Alberta

a Experiment 1: Effect of the Glucosinolate Content of Rapeseed Meal on the Incidence of Fishy Egg Production by Brown-Shelled Egg Layers

1 Objectives

This experiment was designed to examine the effects of glucosinolate levels in RSMs fed at 10% of the laying ration to brown-shelled egg layers on the production of fishy eggs. The relationship between the concentration of TMA found in the egg and the intensity of fishy odor was also investigated.

2 Materials and Methods

A flock of 400 Rhode Island Red (RIR) layers was screened for fishy egg layers. This was done by determining which of the birds laid eggs with a fishy odor when fed a laying ration containing 10% of high glucosinolate RSM and not when fed a laying ration containing no RSM. Sensory evaluation of egg odor was carried out by a panel of four testers. Starting on the second day after the birds were placed on the RSM-containing ration all eggs produced during the subsequent two weeks were broken and the inside of the shell was smelled for fishy odor. The degree of odor was scored on a five point scale from normal (0) to strongly fishy (4). Those birds that laid at least three fishy eggs when fed the RSM-containing ration were then fed a laying ration containing no RSM. After a lapse of two weeks on the ration devoid of RSM, the eggs laid during the subsequent ten days were evaluated for fishy odor. By this

screening process 40 birds were selected which laid fishy eggs when fed the ration containing RSM but laid no fishy eggs when fed the ration devoid of RSM.

The 40 selected birds were divided into four groups of 10 birds and each group was fed one of the test rations shown in Table 33. Briefly, the rations consisted of a control laying ration devoid of RSM and laying rations containing 10% of high glucosinolate RSM (HG-RSM), 10% of medium glucosinolate RSM (MG-RSM) or 10% of low glucosinolate RSM (LG-RSM). In addition to these four rations, three other rations (Rations 5, 6 and 7) were prepared by adding 3% of ground raw rapeseed to each of rations 2, 3 and 4.

The birds were caged individually and egg collection for sensory evaluation began on the tenth day after the birds were started on the test rations and continued for 14 days. The last three eggs laid by each bird on each ration were sensory evaluated and the egg contents were kept for TMA analysis.

After these egg odor evaluations had been completed, the three groups of birds that had received rations 2, 3 and 4, containing 10% RSM were, respectively switched to rations 5, 6 and 7, containing 10% RSM plus 3% ground raw rapeseed, while the control group was continued on ration 1. Egg collection over the next eight days were sensory evaluated. The egg contents of the last three eggs laid by each bird on each of the four rations were kept for TMA analysis.

Table 33

Composition of the Experimental Rations[1]

Ingredients	Rations 1 Control SBM	 2 10% HG-RSM	 3 10% MG-RSM	 4 10% LG-RSM
Ground wheat	52.115	52.115	52.115	52.115
Ground oats	10	10	10	10
Ground barley	10	10	10	10
Wheat shorts	5.0	0	0	0
Stabilized fat	1.0	2.0	2.0	2.0
Dehydrated alfalfa	2.0	2.0	2.0	2.0
Meat meal (55% protein)	2.0	2.0	2.0	2.0
Herring meal (72% protein)	1.0	1.0	1.0	1.0
HG-RSM[2]	-	10	-	-
MG-RSM[3]	-	-	10	-
LG-RSM[4]	-	-	-	10
Soybean meal (48.5% protein)	8.0	2.0	2.0	2.0
Ground limestone	6.0	6.0	6.0	6.0
Calcium phosphate (18.5% Ca - 20.5% P)	1.5	1.5	1.5	1.5
Iodized salt	0.45	0.45	0.45	0.45
Manganous oxide	0.025	0.025	0.025	0.025
Zinc oxide	0.01	0.01	0.01	0.01
Micronutrient mix[5]	0.9	0.9	0.9	0.9

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[1] Three additional rations (Rations 5, 6 and 7) were prepared by adding 3% of raw ground rapeseed (*B. campestris* var. Span) to each of rations 2, 3 and 4.

[2] High glucosinolate rapeseed meal (*B. napus* mixed varieties).

[3] Medium glucosinolate rapeseed meal (*B. napus* var. Turret).

[4] Low glucosinolate rapeseed meal (*B. napus* var. Tower).

[5] Supplied the following levels per kg of ration: Vitamin A, 6000 IU; Vitamin D_3, 120 ICU; riboflavin, 3 mg; calcium pantothenate, 6 mg; niacin, 15 mg; Vitamin B_{12}, 6.6 ug and penicillin, 4.4 mg.

When egg contents were retained for TMA analysis, the eggs were broken into a beaker, homogenized, sealed in a plastic bag and stored at -20°C until analysed. The amount of TMA in the eggs produced was determined by the method of head space gas analysis by gas liquid chromatography of Metha *et al*. (1974) and the procedure for extracting the TMA from egg content was a modification of that of Hobson-Frohock *et al*. (1973). The analytical procedure for TMA is described in the Appendix.

The glucosinolate contents of the three RSMs used in this study were determined by the method of Appelqvist and Josefsson (1967) and were expressed as their potential hydrolytic products, isothiocyanate and oxazolidinethione.

3 Results and Discussion

The data recorded when the birds were fed the control and RSM-containing laying rations are summarized in Table 34. The TMA concentrations and fishy odor scores shown were the means for the various groups of birds receiving the test rations. In calculating the average fishy odor scores in this table, only the scores of the three eggs kept from each bird for TMA analysis were considered.

Despite the prescreening process to which these birds were subjected to detect potential to lay fishy eggs when fed a RSM-containing ration, some of the birds ceased to lay eggs with a fishy odor or produced eggs with only a faintly fishy odor when they were transferred from floor pens to cages and placed on the RSM-containing experimental rations. Three of

Table 34

Effects of Glucosinolate Contents of Rapeseed Meal on the Trimethylamine Content and Fishy Odor Score of Eggs

	Rations				
	1	2	3	4	
	Control SBM	10% HG-RSM	10% MG-RSM	10% LG-RSM	S.E.[1]
Glucosinolate					
Isothiocyanate, mg/g meal	N.D.[2]	3.75	1.39	0.39	
Oxazolidinethione, mg/g meal	N.D.	9.13	3.36	1.13	
TMA, ug/g egg[3]	0	0.74^{a4}	0.53^{a}	0.71^{a}	±0.24
Fishy odor score[3]	0	1.26^{a}	0.46^{b}	0.56^{b}	±0.16
Total number of egg tested	103	96	97	96	
Percent fishy egg detected	0	71.9	37.1	14.6	

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[1] Standard error of the mean with n = 9.

[2] Not determined.

[3] Data recorded for the control ration were not included in the statistical analysis.

[4] Means on the same row with the same superscript were not significantly different ($P<0.05$).

the birds were out of production by the time egg collection for odor evaluation began. In the sensory evaluations by the panel of testers, an egg was considered as fishy when it was scored fishy by three or more of the panel of four regardless of the intensity of odor. Based on this criterion, the percentages of fishy eggs detected were 71.9, 37.1 and 14.6 for the groups receiving 10% HG-RSM, MG-RSM and LG-RSM in the rations, respectively. Eggs produced by birds on the control ration were found to be completely free of fishy odor. These results appeared to relate positively the laying of fishy eggs with the level of glucosinolates in the RSM included in the laying ration. However, when the data for TMA content of eggs and fishy odor score for the three groups receiving the RSM-containing rations were analysed by one-way analysis of variance and significant differences between means were compared by Duncan's Multiple Range test (Steel and Torrie, 1960) no significant trend for increased TMA content of eggs with increasing levels of glucosinolate in the RSM contained in the ration was observed. The lack of effect of glucosinolate levels in the meals on TMA contents of the eggs suggested that factor(s) other than glucosniolates in RSMs must be responsible for the deposition of TMA found in the eggs.

The effects of inclusion of 3% of raw ground rapeseed to the RSM-containing laying rations on the occurrence of fishy eggs are summarized in Table 35. The percentage of fishy eggs detected by the panel was slightly reduced in the groups receiving the rations containing 10% HG-RSM and MG-RSM and

Table 35

Effect of Inclusion of Ground Raw Rapeseed in Rations Containing Rapeseed Meal on the Trimethylamine Content and Fishy Odor Score of Eggs

	Rations				
	1	5	6	7	
	Control SBM	10% HG-RSM	10% MG-RSM	10% LG-RSM	S.E.[1]
Raw rapeseed, %	0	3	3	3	
TMA, ug/g egg[2]	0	3.01^{a}[3]	1.79^{b}	2.74^{ab}	±0.30
Fishy odor score[2]	0	0.62^{a}	0.13^{b}	0.43^{ab}	±0.10
Total number of egg tested	58	48	60	60	
Percent fishy egg detected	0	62.5	25	46.7	

[1] Standard error of the means with n = 9.

[2] Data recorded for the control ration were not included in the statistical analysis.

[3] Means on the same row with the same superscript were not significantly different ($P<0.05$).

slightly increased in the group fed the ration containing LG-RSM when compared to the results obtained when the same RSMs were fed in the absence of raw ground rapeseed (Table 35 vs Table 34).

The addition of 3% ground raw rapeseed to the RSM-containing laying rations appeared to increase the deposition of TMA in the eggs produced (Table 35 vs Table 34). The TMA levels found in most of the homogenized egg samples were above 1 ug/g egg (ranged from 0.26 to 5 ug/g) and the means were 3.01, 1.79 and 2.74 ug/g egg for the three groups of birds fed the rations containing 10% HG-RSM, MG-RSM and LG-RSM respectively. The means ranked in the same order as when the birds were fed the same RSM in the absence of ground raw rapeseed in the ration. The fishy odor scores of the eggs used in the TMA analysis agreed with the results obtained in the TMA analysis (Table 35). When the TMA and fishy egg score data were analysed statistically, it was found that with respect to both traits the values for the HG-RSM were significantly higher than the values for the MG-RSM but not different from values for the group fed the ration containing LG-RSM ($P<0.05$).

Unheated ground rapeseed is known to contain the enzyme myrosinase which hydrolyses glucosinolates to yield their primary end-products, isothiocyanates and oxazolidinethione, hence the addition of 3% ground raw rapeseed in the second half of this study maximized the possible effects of glucosinolate hydrolysis products on the production of eggs with a fishy odor. The data obtained on the TMA content and fishy

odor score of eggs laid by the birds fed such rations indicated no relationship between these traits and the level of glucosinolate hydrolysis products. This finding lends further support to the earlier suggestion that a factor(s) other than glucosinolates is responsible for the production of fishy odor in eggs laid by brown-shelled egg layers fed rations containing RSM.

b Experiment 2: Effect of Supplementing a Laying Ration with Choline on the Production of Fishy Eggs by Brown-Shelled Egg Layers

1 Objective

This experiment was undertaken to study the effect of supplementing a soybean meal (SBM) type laying ration with choline to a level equivalent to that of a ration containing 10% RSM on the production of fishy eggs by brown-shelled egg layers.

2 Materials and Methods

Forty Rhode Island Red layers selected from another flock of birds by a screening process similar to that followed in the previous experiment were used in this study. The selected birds were divided into four comparable groups of ten birds each and fed the rations referred to below.

Four experimental rations were used. Three of the rations were prepared by supplementing the SBM control ration used in Experiment 1 with choline chloride at rates of 0, 250 and 500 mg choline per kg ration. The fourth ration was identical to ration 2 used in the previous experiment which contained 10% of high glucosinolate RSM (HG-RSM). The highest level of choline supplementation would raise the calculated choline content of the control ration to 1500 mg per kg ration which was similar to that calculated for the HG-RSM containing ration.

Egg collection for sensory evaluation began on the tenth day after the birds were on the experimental rations and eggs

collected over eight days were evaluated. Sensory evaluation of the fishy odor of the eggs produced by the birds on the experimental rations was conducted in a manner similar to that described in the previous experiment. The last three eggs laid by individual birds during the sensory evaluation period were pooled and analysed for TMA content.

3 Results and Discussion

The results obtained in the present experiment are summarized in Table 36. None of the eggs from the groups of birds fed the control laying ration or the control ration supplemented with choline contained measurable amounts of TMA or smelled fishy. In contrast, the eggs from the group of birds fed the ration containing 10% HG-RSM contained appreciable amounts of TMA (0.26 to 4.22 ug/g raw egg with a mean of 2.07) and were graded fishy by the panel of testers. The average odor score was 1.56 which means that the eggs were slightly to moderately fishy. Ignoring the intensity of the fishiness, 67% of the eggs laid by this group of birds were judged as fishy.

The results obtained in the present study clearly demonstrated the lack of effect of supplementary choline on the production of fishy eggs by the brown-shelled egg layers. Even at the highest level of supplementation, choline chloride failed to induce fishy egg layers to lay eggs with a detectable fishy odor. This observation therefore seems to rule out the high level of choline in RSM as a possible cause of fishy odor in eggs. However, it should be noted that the form in which

Table 36

Effect of Supplementary Choline on the Production of Eggs with a Fishy Odor by Brown-Shelled Egg Layers

	Rations				
Choline added, mg/kg ration	Control[1] 0	Control 250	Control 500	10% HG-RSM 0	S.E.[2]
TMA, μg/g egg	0	0	0	2.07	±0.45
Fishy odor score	0	0	0	1.56	±0.21
Total number of eggs tested	60	62	59	52	
Percent fishy eggs detected	0	0	0	67	

[1] See Table 33, ration 1 for formula.

[2] Standard error of the mean for the group fed 10% HG-RSM, n = 8.

the choline was present in the choline supplemented rations was different from the form in which it was present in the ration containing 10% of HG-RSM. Whereas the choline in the supplemented rations was mainly in free form and thus readily available for absorption, the choline in rapeseed is known to exist in free form or bound as a cationic moiety of the phospholipid, lecithin, or bound to sinapic acid to form the ester sinapine. Most of the phospholipid in rapeseed is removed during the oil extraction process (Weenink and Tulloch, 1966) but the sinapine remains in the meal fraction. RSM contains approximately 1% of the sinapine (Austin and Wolff, 1968).

If the TMA which found its way into eggs was in fact derived from the breakdown of choline by the microbial action of intestinal bacteria, free choline, because of its availability for rapid absorption, would less likely be converted to TMA by intestinal bacteria than choline in a bound form such as in sinapine. The probable effects of phospholipids and sinapine in RSM on the production of fishy eggs by brown-shelled egg layers were not investigated in the present study.

D General Discussion

Since the identification of TMA as the compound responsible for the fishy odor of eggs produced by brown-shelled egg layers fed RSM-containing laying rations by Hobson-Frohock _et al_. (1973), neither the precursor(s) in the meal nor the mechanism which leads to the formation of TMA and eventual deposition in the egg has been elucidated. Several organic

constituents in RSM have been investigated as possible causes of fishy odor in eggs produced by brown-shelled egg layers but results obtained were either inconclusive or failed to show any correlation between the constituent and the intensity of fishy odor of the eggs produced (Vogt *et al*., 1969; Hobson-Frohock *et al*., 1975).

The level of glucosinolates in RSM and the effect of supplementing a laying ration devoid of RSM with choline chloride on the production of fishy odor in eggs produced by brown-shelled egg layers were studied herein. From the results obtained there was no evidence that the use of low glucosinolate RSM significantly reduced the incidence of fishy eggs produced as judged by the amount of TMA found in eggs and fishy odor score. This observation was in agreement with the findings reported by Vogt and Torges (1976). They reported that the use of low glucosinolate RSM (*B*. *napus* var. Erglu) at levels of 7.5 and 15% of the ration caused brown-shelled egg layers to lay eggs, 10 and 26% of which, respectively, had a bad smell and taste.

The presence of 3% ground raw rapeseed in the RSM-containing rations increased the TMA level in eggs as compared to that of eggs laid by birds fed rations without ground raw rapeseed but not the degree of fishy odor. This discrepancy might be attributed to the method of sensory evaluation adopted in the present study or to the changes in microflora in the gut of the birds. In regard to the method of sensory evaluation, Hobson-Frohock *et al*., (1973) reported that the TMA in fishy eggs was concentrated mainly in the yolk rather

than in the albumen. In the present study, the fishy odor of eggs was scored by smelling the broken shells, the odor of which would likely be affected mainly by odor associated with the albumen. On the other hand, TMA values of egg contents would be affected mainly by the TMA in the yolk of the egg and to a lesser extent by the TMA in the albumen. In view of this, one might expect that TMA assay of egg contents would be a more accurate test for fishy eggs than sensory evaluation of the shell odor. Furthermore marked changes in the TMA content of egg yolk might not be reflected in similar changes in the TMA content of the albumen. Under such circumstances one might not expect changes in the TMA in egg contents to be closely correlated with shell odor.

The large variation in the TMA content of eggs from fishy egg layers on the same ration treatment leads one to speculate that several factors are involved. It suggests that even amongst fishy egg layers some birds are able to metabolize the TMA better than others. It also suggests, that if bacteria in the intestines are involved, as suggested earlier, in TMA production from a precursor such as sinapine, the variation in numbers and kinds of bacteria present in the intestines of different birds may be in part responsible for the variation in TMA noted in eggs of fishy egg layers fed the same ration.

The effect of supplementary choline on fishy egg production by the brown-shelled egg layers have already been discussed.

E Summary

Two experiments were undertaken to study the effects of glucosinolate levels in RSM and supplementary choline on the production of fishy eggs by brown-shelled egg layers.

In the first experiment, rapeseed meals of high, medium and low glucosinolate contents were included in laying rations at the 10% level and fed to fishy egg layers for a period of 23 days. Eggs produced were rated for fishy odor intensity beginning on the tenth day on ration and the last three eggs laid by individual birds were pooled and analysed for TMA. It was found that the mean TMA level and mean fishy odor score were numerically highest in the eggs produced by birds fed the ration containing 10% of high glucosinolate RSM but the values obtained were not significantly different from those resulting from the feeding of similar birds a ration containing 10% of low glucosinolate RSM. Supplementing the rations with 3% ground raw rapeseed as a source of myrosinase to hydrolyse the glucosinolates in the RSM produced results similar to those obtained with the unsupplemented RSMs. Thus, on the basis of the results obtained, it seems highly improbable that the level of glucosinolate in RSM affects the incidence of laying of eggs with a fishy odor by brown-shelled egg layers.

In the second experiment a laying ration devoid of RSM was supplemented with 0, 250 and 500 mg choline per kg of ration and fed to fishy egg layers. A fourth group of birds was fed a ration containing 10% high glucosinolate RSM. All

of the eggs produced by the birds fed the ration devoid of RSM, with and without choline supplementation, were rated free of fishy odor and all the eggs analysed from these treatments were found to be free of TMA. In contrast to these observations, eggs produced by the birds fed the 10% RSM-containing ration were tainted with a fishy odor of varying intensity and contained TMA. Thus, free choline does not appear to be involved in the laying of fishy eggs by brown-shelled egg layers.

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APPENDIX

DETERMINATION OF TRIMETHYLAMINE BY GAS LIQUID CHROMATOGRAPHY

A Extraction of Trimethylamine

The frozen egg samples were thawed in a cold room (0°C) overnight and rehomogenized. Approximately 50 g of egg was transferred into a 250 ml round bottom flask with ground glass joint and frozen by immersing the flask in liquid nitrogen (-198°C). After freezing was completed the flask was immediately connected to a inverted U shaped tube with ground glass joints and a side outlet connected to a vacuum pump. A 100 ml collection flask was connected by ground glass joint to the other end of the U-tube. While the flask containing the egg sample was in liquid nitrogen, the air in the system was evacuated. Next, the stopper in the side outlet leading to a vacuum pump was turned to the closed position. The liquid nitrogen container was then transferred to cool the collection flask. Low heat was applied to the flask containing the frozen egg sample by immersing it in warm water bath. The freeze drying was stopped when about 50% of the egg sample was collected in the collecting flask. The frozen condensate was allowed to thaw and acidified with diluted HCl to pH 3 and stored when necessary in a freezer at -20°C. The trimethylamine hydrochloride salt was recovered by freeze-drying the condensate. It was then dissolved in 2 ml of de-ionized water and analysed for TMA by head space gas chromatography.

B Head Space Gas Analysis by Gas Liquid Chromatography

A gas liquid chromatograph (Aerograph Hy Fi Model 600D) equipped with a flame ionization detector was used for the head space gas analysis and the chromatogram was recorded on a Fisher Recorder (Fisher Recordall Series 5000). A glass column (3.05 m x 3.18 mm) was packed with Penwalt 231 GC packing (Applied Science Lab. Inc.). The column was preconditioned at 150°C for 24 hours before use.

Samples were prepared for GLC analysis according to the procedure (Metha et al., 1974) used for the determination of TMA in milk. An aliquot of 0.5 ml of the concentrated condensate containing the TMA·HCl obtained by vacuum distillation of the egg sample was transferred to a 3 ml serum tube and diluted to 1 ml with distilled water. The tube was then evacuated by withdrawing 10 ml of the air by a syringe. One ml of 45% KOH was injected into the tube with a plastic syringe and the tube was shaken for 1 minute to liberate the volatile TMA gas from the solution. 0.5 ml of the head space gas was injected into the GLC. Operation conditions of the GLC were as follows: column temperature, 85°C; detector temperature, 85°C; injector temperature, 140°C; hydrogen gas flow rate, 30 ml per minute; air flow rate, 350-400 ml per minute; nitrogen (carrier gas) flow rate, 35 ml per minute. Duplicate 0.5 ml samples of the condensate from each pooled egg sample were analysed.

A standard curve relating peak heights of chromatograms and TMA concentrations ranging from 5 to 40 ppm was prepared

by diluting quantities of a stock solution containing 100 ppm TMA to desired concentrations. The appropriately diluted solutions were analysed for TMA in the manner referred to above.

The TMA concentration in the egg samples was expressed in ug/g homogenized egg.

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